ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA

C IMMUNOLOGY

VOL. 85C FASC. 1 FEBRUARY 1977

MUNKSGAARD COPENHAGEN

ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA

Founded 1924

Acta Pathologica et Microbiologica Scandinavica is intended for the prompt publication of original research in the fields of pathology microbiology and immunology It is included in Current Contents, Excerpta Medica, and Medlars.

Acta Pathologica et Microbiologica Scandinavica is a nonprofit making scientific journal. *Since 1924 it has been published by the Scandinavian Societies for Medical* Microbiology and Pathology It appears in three sections Section A Pathology Section B Microbiology, and Section C Immunology

Acta Pathologica et Microbiologica Scandinavica has subscribers in more than seventy countries throughout the world with a wide readership in the major research institutes, hospitals, laboratories, and specialist libraries.

EDITORIAL CORRESPONDENCE

All communications regarding manuscripts and editorial matters should be addressed to the Editorial Office, c/o Inger Danielsen, Secretary, Johnstrups Allé 6, DK 1923 Copenhagen V, Denmark.

SUBSCRIPTION

At present one annual volume of Section A, one of Section B, and one of Section C (each *section consisting of 6 issues appearing bimonthly*) will contain a total of approximately 1600 pages. During the past few years approximately five free supplements have been issued annually These supplements will be delivered separately to the subscribers by surface mail at no extra charge. The subscription price is

Sect. A, B, and C	D.kr 770.– plus postage D.kr 40.– ($ 141 75 £ 86.70 DM 336.15)
Sect. A	D.kr 462.– plus postage D.kr 15.– ($ 83.50, £ 51 05 DM 198.00)
Sect. B	D.kr 462.– plus postage D.kr 15.– ($ 83.50 £ 51.05 DM 198.00)
Sect. C:	D.kr 308.– plus postage D.kr 10.– ($ 55.65 £ 34 10 DM 132.00)
Sect. A and C (combined):	D.kr 660.– plus postage D.kr 25.– ($ 119.90 £ 73.30, DM 284.30)
Sect. B and C (combined)	D.kr 660.– plus postage D.kr 25.– ($ 119.90, £ 73.30 DM 284.30)

Back numbers (whole volumes or single copies) are available.
NB. All prices are subject to exchange rate fluctuation.

All business communications regarding subscriptions, distribution, changes of address, advertisements, or orders of back numbers should be addressed to MUNKSGAARD International Publishers Ltd., 35 Nørre Søgade, DK 1370 Copenhagen K, Denmark.

Second class postage paid at New York, N Y., U.S.A. U.S. mailing agent, Expediters of the Printed Word, Ltd., 527 Madison Avenue, New York, N Y 10022. Printed in Denmark.

ACTA
PATHOLOGICA
ET MICROBIOLOGICA
SCANDINAVICA

INDEX
VOL. 83 C

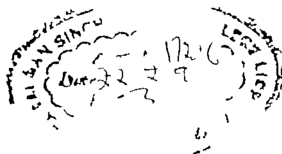
ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA

Section C IMMUNOLOGY

VOL. 85 C. FASC. 1-6 1977

MUNKSGAARD COPENHAGEN

Published by the
Scandinavian Societies for Microbiology and Pathology



Distributed by Munksgaard
International Booksellers and Publishers, Ltd.
35 Nörre Sögade, DK 1370 Copenhagen K, Denmark

INDEX

VOL. 85 C. FASC. 1–6 1977

SUPPLEMENTS

Supplement 257 *Jørgensen Mogens Jørgen* The Ductal Plate Malformation. Pp. 88. 1977 (Section A)

Supplement 258 *Boettcher B, Hjort T., Rümke Ph., Shulman S. and Vyazov O. E.* Auto- and Iso-Antibodies to Antigens of the Human Reproductive System. I. Results of an International Comparative Study of Antibodies to Spermatozoa and other Antigens Detected in Sera from Infertile Patients Deposited in the WHO Reference Bank for Reproductive Immunology. Pp. 69. 1977 (Section C)

Supplement 259 *Mårdh Per-Anders and Rune Grubb* Applications of Gas and High-pressure Liquid Chromatography in Microbiology. A Swedish Society for Microbiology Symposium. Pp. 70. 1977 (Section B)

Supplement 260 *Berge Thorbjörn and Lundberg, Sten* (with clinical comments by *Haeger Knut and Hällén, Jan*) Cancer in Malmö 1958–1969. An autopsy study. Pp. 235. 1977 (Section A)

Supplement 261 *Clemmesen Johannes* Statistical Studies in the Aetiology of Malignant Neoplasms V. Trends and Risks, Denmark 1943–77. Pp. 286. 1977 (Section A)

Supplement 262 *Høiby Niels* Pseudomonas Aeruginosa Infection in Cystic Fibrosis. Diagnostic and Prognostic Significance of Pseudomonas aeruginosa Precipitins Determined by Means of Crossed Immunoelectrophoresis. A Survey. Pp. 96. 1977 (Section C)

Supplement 263 *Nilsson Ole* Studies on the Ultrastructure of the Human Parathyroid Glands in Various Pathological Conditions. Pp. 88. 1977 (Section A)

SUPPLEMENTS

Supplement 257 — *Jørgensen Mogens Jørgen* The Ductal Plate Malformation. Pp 88. 1977 (Section A)

Supplement 258 — *Boettcher B Hjort T., Rümke Ph., Shulman S and Vyazov O E.* Auto- and Iso-Antibodies to Antigens of the Human Reproductive System. 1 Results of an International Comparative Study of Antibodies to Spermatozoa and other Antigens Detected in Sera from Infertile Patients Deposited in the WHO Reference Bank for Reproducti. Immunology Pp. 69 1977 (Section C)

Supplement 259 — *Mårdh Per Anders and R as Grubb* Applications of Gas and High-pressure Liquid Chromatography in Microbiology A Swedish Society for Microbiology Symposium. Pp 70. 1977 (Section B)

Supplement 260 — *Berge Thorbjörn and Lundberg, S en* (with clinical comments by *Haeger K rl and Hällén Jan)* Cancer in Malmö 1958–1969 An utopsy study Pp. 235. 1977 (Section A)

Supplement 261 — *Clemmesen Johannes* Statistical Studies in the Aetiology of Malignant Neoplasms. V Trends and Risks, Denmark 1943–77 Pp. 286. 1977 (Section A)

Supplement 262 — *Høiby Niels* Pseudomonas Aeruginosa Infection in Cystic Fibrosis. Diagnostic and Prognostic Significance of Pseudomonas aeruginosa Precipitins Determined by Means of Crossed Immunoelectrophoresis. A Survey Pp. 96 1977 (Section C)

Supplement 263 — *Nilsson Olav* Studies on the Ultrastructure of the Human Parathyroid Glands in Various Pathological Conditions. Pp 88. 1977 (Section A)

Acta path microbiol scand. Sect. C, 85 1–9 1977

GRANULOCYTE FUNCTION IN BACTERIAL INFECTIONS IN MAN

KJELL B. HELLUM and CLAUS O SOLBERG

The University of Bergen, School of Medicine Medical Department B, Bergen, Norway

Hellum, K. B. & Solberg, C. O Granulocyte function in bacterial infections in man. Acta path. microbiol. scand. Sect C, 85 1–9 1977

The reduction of nitroblue tetrazolium (NBT) dye and the phagocytic and bactericidal activities of neutrophil granulocytes from 141 patients with bacterial infections and 141 controls have been examined and related to granulocyte morphology In 115 patients (82 per cent) the NBT reduction capacity was higher than in any control. Vacuolization of the cytoplasm and/or toxic granulation of the neutrophils were demonstrated more often in patients with high than low NBT reduction capacity In 49 patients (35 per cent) the bactericidal activity of the neutrophils was lower than in any control. Thirty-eight patients (78 per cent) with impaired bactericidal activity had 25 per cent or more peripheral juvenile neutrophils as compared with only 12 (13 per cent) out of 92 patients with normal activity Vacuolization of the cytoplasm and/or toxic granulation of the neutrophils were demonstrated in 28 patients (57 per cent) in whom the granulocyte function was reduced and in 26 patients (28 per cent) in whom function was normal. Within wide limits, the NBT reduction capacity increased with diminishing bactericidal activity of the neutrophils. Eighteen patients died of infection 12 had reduced bactericidal activity Defects in neutrophil granulocyte function caused by bacterial infection may contribute to a fatal outcome of the disease.

Key words Bacterial infections human granulocytes nitroblue tetrazolium reduction phagocytosis bactericidal activity; lethality

K B Hellum, Medical Department B, 5016 Haukeland sykehus, Bergen, Norway

Received 11 I 76 Accepted 5 III 76

Neutrophil granulocytes from patients with bacterial infections frequently show a shift to the left, vacuolization of the cytoplasm, toxic granulation and increased capacity to reduce the yellow nitroblue tetrazolium (NBT) dye to intracellular dark blue formazan. In a previous study we also observed that the bactericidal activity of the granulocytes from patients with severe bacterial infections was frequently reduced, sometimes to such an extent that it might contribute to a fatal outcome of the disease (20) However the reason for this impaired phagocyte function is poorly understood but may be related to changes in granulocyte morphology (8, 20)

In the present study granulocyte morphology formazan production and the phagocytic and bactericidal activities of neutrophil granulocytes have been examined in 141 patients with severe bacterial infections.

MATERIALS AND METHODS

Patients and Controls

Leucocytes were obtained during the acute febrile course in 141 patients with bacterial infec

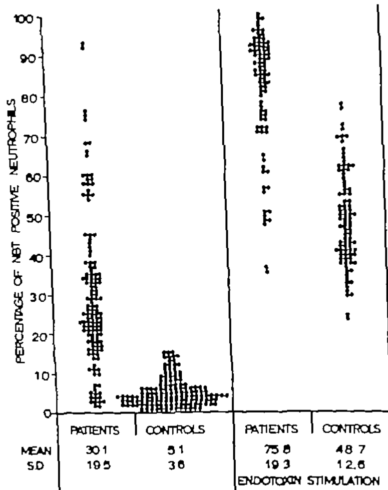


Fig 1 Nitroblue tetrazolium (NBT) test in 141 patients with bacterial infections and 141 normal controls, and endotoxin stimulated NBT test in the last 92 patients and controls.

mycin, 500 units penicillin G and 2 mg phenylbutazone were incubated at 37 C for 15 minutes and centrifuged for 10 minutes at 500 g The cellular pellet was twice washed in 5 ml HBSS by centrifugation at 500 g for 10 minutes and resuspended in 1 ml distilled water to allow osmotic disruption of the leucocytes to occur Quantitation of viable bacteria was made by the standard pour-plate technique.

The bactericidal capacity of the granulocytes is proportional to the total number of bacteria killed and inversely proportional to the total number of viable bacteria or number of viable intracellular bacteria (19) The number of phagocytized bacteria equals the number of viable intracellular bacteria plus the number of bacteria killed.

RESULTS

NBT Test

In the control group, the percentage of NBT positive neutrophils ranged from 1 to 15 mean 5 1 and in the patient group from 2 to 95 mean 30.1 (Fig. 1) Fifty-eight patients presented NBT scores above the mean value and twenty-six patients within the normal range. In the patients with major non-infectious underlying diseases, the percentage of NBT positive neutrophils ranged from 4 to 89 mean 32.0.

Twenty-five per cent or more peripheral juvenile neutrophils were observed in 23 of

TABLE 1 *Diagnosis in 141 Patients with Bacterial Infections Subject to Nitroblue Tetrazolium Test and Granulocyte Function Studies*

Dagnosis	Number of patients
Lower respiratory tract infection	58
Septicaemia	36
Meningitis	18
Urinary tract infection	18
Abscesses and wound infection	11

TABLE 2 *Major Underlying Diseases in 141 Patients with Bacterial Infections Subject to Nitroblue Tetrazolium Test and Granulocyte Function Studies*

Underlying disease	Number of patients
None	102
Cardiovascular	16
Solid cancer	8
Diabetes mellitus	8
Chronic renal and/or liver disease	7

tions (Table 1) admitted to a medical department. Eighty-one were males and 60 females. The ages ranged from 7 to 90 years, mean age was 53 years. Thirty nine patients had major non-infectious underlying diseases (Table 2)

Leucocytes obtained from the medical staff student nurses and medical students in the department were used as controls for comparison of NBT reduction capacity and phagocytic and bactericidal activities. For each test using a patient's leucocytes, a control test was performed. Seventy three of the controls were females and 68 males. The ages ranged from 19 to 66 years mean age being 33 years.

Histochemical NBT Test

The test was performed by a heparinized whole blood (10 units heparin per ml) technique slightly modified from Park's test (15) as previously described (5) In the endotoxin stimulated test 0 5 ml blood was pre-incubated for 10 minutes at 21 C with 100 μg endotoxin (Lipopolysaccharide *E. coli* O26 B6 Difco) in 0 05 ml phosphate buffered saline. The percentage of NBT positive cells (formazan containing) in 300 neutrophils counted in each of 2 slide smears was recorded as the NBT score

Leucocyte-bacteria Suspension (Phagocytosis Test)

Leucocytes. Leucocytes obtained by "Isopaque"/dextran sedimentation of heparinized venous blood (10 units heparin per ml blood) were twice washed in heparinized saline (1 unit heparin per ml saline) by centrifugation at 500 *g* for 5 minutes (19) A differential count was made and the cells were resuspended to 10^7 neutrophils per ml in Hanks balanced salt solution (HBSS) containing 0.1 per cent gelatin The functional integrity of the isolated neutrophils remained intact as measured by latex particle phagocytosis, and 95 to 98 per cent resisted staining with trypan blue. Eosinophil granulocyte contamination in 100 consecutive patient specimens varied from 0 and 5 per cent (mean 2 per cent) basophil granulocyte contamination from 0 to 2 per cent (mean 1 per cent) and lymphocyte—monocyte contamination from 9 to 18 per cent (mean 13 per cent) In 100 consecutive control specimens, eosinophil granulocyte contamination varied from 0 to 7 per cent (mean 3 per cent) basophil granulocyte contamination from 0 to 2 per cent (mean 1 per cent) and lymphocyte-monocyte contamination from 12 to 25 per cent (mean 17 per cent)

Bacteria Staphylococcus aureus "Oxford" (Heatley strain obtained from the National Collection of Type Cultures, Colindale London, 1958) was used as test organism (19) The bacteria were cultured overnight in Penassay broth (Difco) twice washed in 0 45 per cent saline and suspended in HBSS to an optical density of 0 6 at 620 nm in a Beckman spectrophotometer This suspension was diluted in HBSS containing 0 1 per cent gelatin to a concentration of 10–14 × 10^7 colony forming units per ml.

Serum Fresh, pooled serum from six adults was stored at −30 C in 1 ml aliquots. Immediately before each experiment, 1 ml serum was thawed and added to 3 ml HBSS containing 0 1 per cent gelatin.

Test procedure 0.5 ml leucocyte suspension, 0 1 ml bacteria suspension and 0 4 ml diluted serum were added to 12 × 75 mm disposable plastic tubes. This provided 2–3 bacteria per granulocyte and a final serum concentration of 10 per cent. The tubes were incubated at 37 C with an end-over-end rotation to promote contact between bacteria and leucocytes. Samples were removed periodically for determination of the total number of viable bacteria and the number of viable intracellular bacteria.

The total number of viable bacteria was determined after osmotic disruption of the leucocytes by adding 0 01 ml leucocyte bacteria suspension to 1 ml distilled water Quantitation of viable bacteria was made from appropriate dilutions of this suspension, using a standard pour plate technique and Penassay agar (Difco)

The number of viable intracellular bacteria was determined as described earlier (19) 0 01 ml of the leucocyte-bacteria suspension and 1 ml HBSS containing 0 1 per cent gelatin, 500 μg strepto-

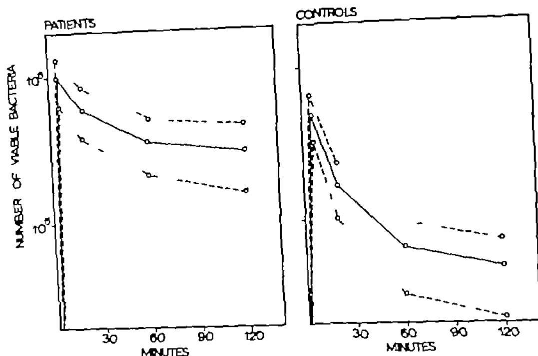


Fig. 3. Number of viable intracellular bacteria during incubation with neutrophil granulocytes from 141 patients with bacterial infections and 141 normal controls.
—— = mean, - - - = ± 1 S.D

cent) of the bacteria remained viable after incubation for 120 minutes (Fig. 2). In contrast, 0.2 to 10.8 per cent (mean 2.6 per cent) of bacteria in the tests using patient neutrophils remained viable after 120 minutes (Fig. 2). At 20, 60 and 120 minutes the mean values for the patient and control groups differed significantly (t-test, $p < 0.005$). In the tests using neutrophils from the patients, large numbers of viable bacteria were located intracellularly (Fig. 3) indicating unimpaired phagocytosis but reduced intracellular killing of bacteria. Again the mean values for the patient and the control groups at 20, 60 and 120 minutes were significantly different (t-test, $p < 0.005$). The differences between the mean values for the patients with and without major non-infectious underlying diseases were minor and not significant (t-test, $p \geqq 0.10$).

In 49 patients, the bactericidal capacity of the neutrophils was lower than in any control, i.e. more than 2.6 per cent of bacteria remained viable after incubation for 120 minutes. Twenty-four of these patients had septicaemia, 14 had lower respiratory tract infection, 6 meningitis, 4 urinary tract infections, and one had liver abscesses.

Twenty five per cent or more peripheral juvenile neutrophils were observed in 38 of the 49 patients in whom the bactericidal capacity was impaired as compared with only 12 of the remaining 92 patients. Vacuolization of the cytoplasm and/or toxic granulation of the neutrophils were prominent findings in 28 patients in whom the granulocyte function was reduced and in 26 of the 92 patients in whom the function was normal.

Thirty-five patients in whom the bactericidal activity was reduced could be followed for 6 days or more after they had become afebrile. Neutrophil granulocyte function became normal within these first 6 days.

In 12 of the 18 patients who died from their infections, the bactericidal capacity was reduced, i.e. 3.8 to 10.8 per cent of the test

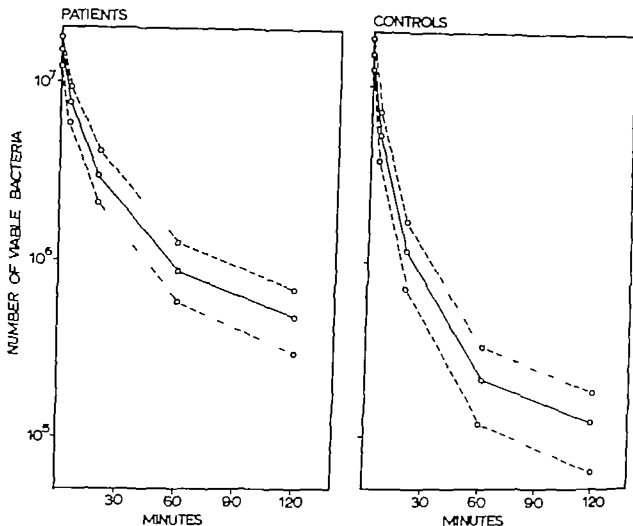


Fig 2 Total number of viable bacteria during incubation with neutrophil granulocytes from 141 patients with bacterial infections and 141 normal controls.
—— = mean. - - - = ± 1 S.D

the 58 patients with NBT scores above the mean value and in 27 of the remaining 83 patients. Vacuolization of the cytoplasm and/or toxic granulation of the neutrophils were characteristic findings in 33 patients with NBT scores above the mean value and in only 21 of the remaining 83 patients.

Seventy two patients with high NBT scores (above the score for any normal control) were followed for 6 days after institution of therapy Within this period the number of NBT positive neutrophils returned to normal in 62 patients. In the remaining 10 patients, severe symptoms of infection persisted.

Endotoxin stimulated tests were performed in the last 92 patients and controls. The percentage of NBT positive neutrophils in the patient group ranged from 22 to 99 mean 75 8, and in the control group from 23 to 76, mean 48.7 (Fig 1)

Twenty-nine patients died—18 from their infections, 4 from cancer with widespread metastases, 3 from cerebral haemorrhage 2 from pulmonary emboli 1 from cardiac insufficiency and 1 from uraemia The mean NBT value (unstimulated) for the 18 patients who died from infection was 35.3 per cent.

Phagocytosis Test

In the tests with neutrophils from the normal controls, 0 1–2 6 per cent (mean 1 0 per

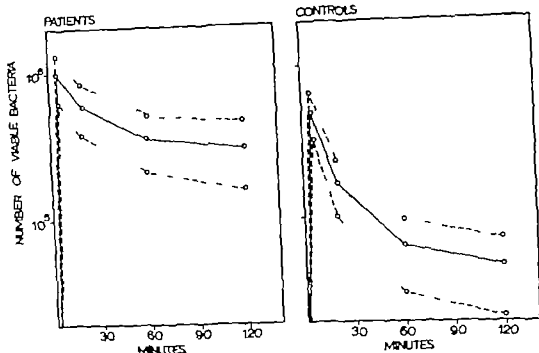


Fig. 3 Number of viable intracellular bacteria during incubation with neutrophil granulocytes from 141 patients with bacterial infections and 141 normal controls.
—— = mean, - - - = ± 1 S.D

cent) of the bacteria remained viable after incubation for 120 minutes (Fig. 2) In contrast, 0.2 to 10.8 per cent (mean 2.6 per cent) of bacteria in the tests using patient neutrophils remained viable after 120 minutes (Fig 2) At 20 60 and 120 minutes the mean values for the patient and control groups differed significantly (t test, $p < 0.005$) In the tests using neutrophils from the patients, large numbers of viable bacteria were located intracellularly (Fig. 3) indicating unimpaired phagocytosis but reduced intracellular killing of bacteria. Again the mean values for the patient and the control groups at 20 60 and 120 minutes were significantly different (t-test, $p < 0.005$) The differences between the mean values for the patients with and without major non-infectious underlying diseases were minor and not significant (t test, $p \geq 0.10$)

In 49 patients, the bactericidal capacity of the neutrophils was lower than in any control i.e. more than 2.6 per cent of bacteria remained viable after incubation for 120 minutes. Twenty four of these patients had septicaemia, 14 had lower respiratory tract infection, 6 meningitis, 4 urinary tract infections, and one had liver abscesses.

Twenty five per cent or more peripheral juvenile neutrophils were observed in 38 of the 49 patients in whom the bactericidal capacity was impaired as compared with only 12 of the remaining 92 patients. Vacuolization of the cytoplasm and/or toxic granulation of the neutrophils were prominent findings in 28 patients in whom the granulocyte function was reduced and in 26 of the 92 patients in whom the function was normal.

Thirty-five patients in whom the bactericidal activity was reduced could be followed for 6 days or more after they had become afebrile. Neutrophil granulocyte function became normal within these first 6 days.

In 12 of the 18 patients who died from their infections, the bactericidal capacity was reduced, i.e. 3.8 to 10.8 per cent of the test

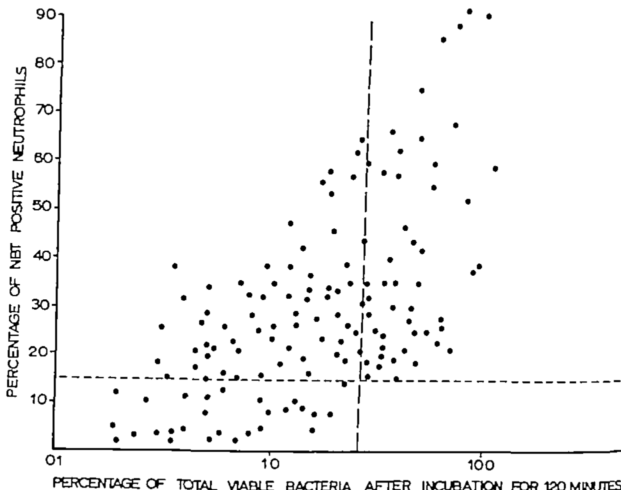


Fig 4 Relationship between results of the nitroblue tetrazolium (NBT) test and the phagocytosis test in 141 patients with bacterial infections.
– – – = upper level of NBT test results in 141 normal controls (see text)
— — = upper level of phagocytosis test results in 141 normal controls (see text)

bacteria remained viable after incubation for 120 minutes.

Relationship between NBT and Phagocytosis Tests

Within wide limits the NBT scores increased with *increasing numbers of viable* intracellular bacteria in the phagocytosis test (Fig 4) The correlation coefficient was 0 46.

DISCUSSION

NBT dye reduction by phagocytes depends on the ingestive as well as the oxidative potentials of the cells (13) However the exact mechanisms involved in the reduction of the dye to formazan are unclear There is general agreement that the reduction is coupled to pyridine nucleotide mediated red-ox reactions (2) During phagocytosis, the activity of these reactions is greatly amplified resulting in an increased production of hydrogen peroxide and labile intermediates like superoxide which may be responsible for the NBT dye reduction *within the phagocytic vacuole* (12) The factors responsible for the entry of the dye into the phagocytes also seem complex. However particles, bacterial products, immune complexes, activated complement, acute phase proteins, high heparin concentrations and contact with glass, all are factors which may induce internalization of soluble or complexed NBT through membrane interaction (2 18) Indeed, the reduced dye acts as a visible marker of phagocytosis and the

enhanced phagocytic capacity of the neutrophils, as demonstrated in vitro during bacterial infections (8, 22) seems to form the biological basis for the elevated NBT reduction in this condition (10). Thus, the strong positive correlation between elevated NBT reduction and bacterial infection demonstrated in the present study supports these concepts of NBT dye reduction. However the association was not invariable, in conformity with findings in previous clinical studies using the histochemical NBT test (18). The growing list of false negative and false positive results has also hampered an assessment of the accuracy and usefulness of this test in the differential diagnosis of febrile disorders, particularly since different techniques have been used in several studies (9, 18).

To some extent NBT reduction seems to be related to granulocyte morphology. In our patients, vacuolization of the cytoplasm and/or toxic granulation of the neutrophils were prominent findings in the group presenting elevated NBT scores, confirming the positive correlation between toxic neutrophils and increased formazan formation in the histochemical test (11). In a previous study it was also demonstrated that juvenile and immature neutrophils are less able to reduce the NBT dye (18). In our study however there was no significant correlation between NBT reduction and a "shift to the left" in the peripheral blood, and even increased formazan production was demonstrated in several patients with large numbers of juvenile and immature neutrophils. Possibly the impaired NBT reduction by a subpopulation of younger cells was more than compensated by the remaining population, resulting in an overall enhanced dye reduction. On the other hand, cellular immaturity may not have influenced significantly the formazan production in our patients since enhanced production was demonstrated by the endotoxin stimulated NBT test which is proposed to be a more relevant index of the functional capacity of the neutrophils than the unstimulated test (16, 18).

Phagocytosis and intracellular killing of bacteria by neutrophil granulocytes are essential host-defence mechanisms and defects in either of these functions have been related to disease syndromes characterized by chronic infections, i.e. deficiencies of complement components (23), chronic granulomatous disease (6), myeloperoxidase (7) and glucose-6-phosphate dehydrogenase (4) deficiencies. Furthermore, acquired or transient defects of neutrophil bactericidal capacity other than drug induced defects have been demonstrated in severe trauma and burns (1), in various malignancies including preleukaemia (17, 21), after irradiation (3), in patients with large numbers of toxic neutrophils (11) or severe bacterial infections (8, 20). In a recent study we demonstrated impaired bactericidal capacity despite normal ingestion in about one third of the patients with major bacterial infections (20). The results of the present study, using the same sensitive assay for granulocyte function, confirm and extend our previous findings. The defect was correlated to a "shift to the left" in the peripheral blood smears and to the appearance of vacuolization of the cytoplasm and/or toxic granulation of the neutrophils. The infection ran also a fatal course more frequently in patients with functional impairment.

The reason for the reduced granulocyte function in our patients remains obscure. In bacterial infections, however, increased numbers of juvenile and immature neutrophils appear in the peripheral blood and the bactericidal activity of these younger cells seems to be diminished (8). The marked "shift to the left" in the peripheral blood from the majority of our patients in whom the granulocyte function was impaired may therefore have contributed to the impairment. However, other factors may also be of importance since several of our patients in whom the granulocyte function was normal had high numbers of peripheral juvenile neutrophils.

The killing of bacteria by neutrophil granulocytes involves a complex series of biochemical events (7). It is likely that multiple, interrelated killing mechanisms exist within

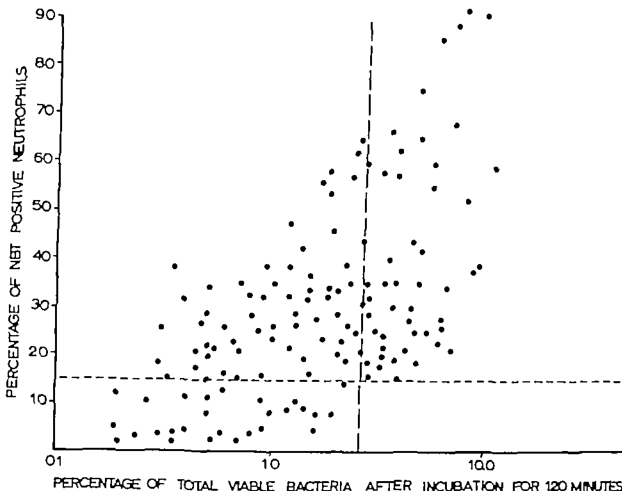


Fig 4 Relationship between results of the nitroblue tetrazolium (NBT) test and the phagocytosis test in 141 patients with bacterial infections.
- - - = upper level of NBT test results in 141 normal controls (see text)
— — = upper level of phagocytosis test results in 141 normal controls (see text)

bacteria remained viable after incubation for 120 minutes.

Relationship between NBT and Phagocytosis Tests

Within wide limits the NBT scores increased with increasing numbers of viable intracellular bacteria in the phagocytosis test (Fig 4) The correlation coefficient was 0 46

DISCUSSION

NBT dye reduction by phagocytes depends on the ingestive as well as the oxidative potentials of the cells (13) However the exact mechanisms involved in the reduction of the dye to formazan are unclear There is general agreement that the reduction is coupled to pyridine nucleotide-mediated red-ox reactions (2) During phagocytosis, the activity of these reactions is greatly amplified resulting in an increased production of hydrogen peroxide and labile intermediates like superoxide which may be responsible for the NBT dye reduction within the phagocytic vacuole (12) The factors responsible for the entry of the dye into the phagocytes also seem complex. However particles, bacterial products, immune complexes activated complement, acute phase proteins, high heparin concentrations and contact with glass, all are factors which may induce internalization of soluble or complexed NBT through membrane interaction (2 18) Indeed the reduced dye acts as a visible marker of phagocytosis and the

enhanced phagocytic capacity of the neutrophils, as demonstrated in vitro during bacterial infections (8, 22) seems to form the biological basis for the elevated NBT reduction in this condition (10) Thus, the strong positive correlation between elevated NBT reduction and bacterial infection demonstrated in the present study supports these concepts of NBT dye reduction. However the association was not invariable, in conformity with findings in previous clinical studies using the histochemical NBT test (18) The growing list of false negative and false positive results has also hampered an assessment of the accuracy and usefulness of this test in the differential diagnosis of febrile disorders, particularly since different techniques have been used in several studies (9 18)

To some extent NBT reduction seems to be related to granulocyte morphology In our patients, vacuolization of the cytoplasm and/or toxic granulation of the neutrophils were prominent findings in the group presenting elevated NBT scores, confirming the positive correlation between toxic neutrophils and increased formazan formation in the histochemical test (11) In a previous study it was also demonstrated that juvenile and immature neutrophils are less able to reduce the NBT dye (18) In our study however there was no significant correlation between NBT reduction and a "shift to the left" in the peripheral blood, and even increased formazan production was demonstrated in several patients with large numbers of juvenile and immature neutrophils. Possibly the impaired NBT reduction by a subpopulation of younger cells was more than compensated by the remaining population, resulting in an overall enhanced dye reduction. On the other hand, cellular immaturity may not have influenced significantly the formazan production in our patients since enhanced production was demonstrated by the endotoxin stimulated NBT test which is proposed to be a more relevant index of the functional capacity of the neutrophils than the unstimulated test (16, 18)

Phagocytosis and intracellular killing of bacteria by neutrophil granulocytes are essential host-defence mechanisms and defects in either of these functions have been related to disease syndromes characterized by chronic infections, i.e. deficiencies of complement components (23) chronic granulomatous disease (6) myeloperoxidase (7) and glucose-6-phosphate dehydrogenase (4) deficiencies. Furthermore, acquired or transient defects of neutrophil bactericidal capacity other than drug induced defects have been demonstrated in severe trauma and burns (1) in various malignancies including preleukaemia (17 21) after irradiation (5) in patients with large numbers of toxic neutrophils (11) or severe bacterial infections (8, 20) In a recent study we demonstrated impaired bactericidal capacity despite normal ingestion in about one third of the patients with major bacterial infections (20) The results of the present study using the same sensitive assay for granulocyte function, confirm and extend our previous findings. The defect was correlated to a "shift to the left" in the peripheral blood smears and to the appearance of vacuolization of the cytoplasm and/or toxic granulation of the neutrophils. The infection ran also a fatal course more frequently in patients with functional impairment.

The reason for the reduced granulocyte function in our patients remains obscure In bacterial infections, however increased numbers of juvenile and immature neutrophils appear in the peripheral blood and the bactericidal activity of these younger cells seems to be diminished (8) The marked "shift to the left" in the peripheral blood from the majority of our patients in whom the granulocyte function was impaired may therefore have contributed to the impairment. However other factors may also be of importance since several of our patients in whom the granulocyte function was normal had high numbers of peripheral juvenile neutrophils.

The killing of bacteria by neutrophil granulocytes involves a complex series of biochemical events (7) It is likely that multiple, interrelated killing mechanisms exist within

each cell and that only some of these are effective against a specific class of microorganisms (7) However following phagocytosis, degranulation of the neutrophils occurs and hydrogen peroxide is relased which, together with the granule enzyme myeloperoxidase and a halide form a strong antimicrobial system, probably the strongest bactericidal system of the granulocytes. In chronic granulomatous disease the bactericidal defect is characterized by an impaired post phagocytic oxygen consumption and hexose monophosphate shunt activity resulting in a reduced hydrogen peroxide generation and inability to reduce intracellular NBT to formazan (17) Other but less severe deficiencies are due to lack of myeloperoxidase (7) In patients with *transient neutrophil dysfunction* like several of our patients, intracellular enzyme defects have not been clearly demonstrated although the defect has been attributed to the azide sensitive myeloperoxidase mediated bactericidal system (8) Moreover low levels of myeloperoxidase-reacting granules have formerly been demonstrated in toxic neutrophils (24) and such neutrophils with vacuolization of the cytoplasm and/or toxic granulation were prominent in several of our patients with neutrophil dysfunction. However neither myeloperoxidase nor any of a number of other intracellular enzyme activities were reduced in a series of extensive studies on toxic neutrophils from patients with acute bacterial infections and other conditions (11) These neutrophils had impaired clearing capacity of *Staph aureus in vitro* in spite of supranormal oxidative metabolism particle ingestion and histochemical NBT dye reduction (11) Accordingly the transient neutrophil dysfunction demonstrated in several of our patients seems neither to be due to reduced hexosemonophosphate shunt activity nor simply to reduced myeloperoxidase levels, but to some other metabolic disturbances of the granulocytes.

Although there was no strong correlation between the reduced bactericidal activity of the neutrophils and the increased NBT reduction in our study these alterations might have a denominator in common. *In vitro* various antigen-antibody complexes may stimulate the phagocytosis and NBT reduction (14 18) Such immune complexes, occurring *in vivo* during infection, could possibly also influence the complex interlocking killing mechanisms of the granulocytes, resulting in impaired bactericidal activity

---

The study was supported by grants from *The Norwegian Research Council for Science and Humanities*

REFERENCES

1 *Alexander J W Windhorst D B & Good R A* Improved tests for the evaluation of neutrophil function in human disease. J Lab clin. Med. *72* 136–148 1968.

2. *Baehner R L* Use of the nitro blue tetrazolium test in clinical pediatrics. Amer J Dis. Child *128* 449–451 1974

3 *Baehner R L Neiburger R G Johnson D E & Murmann S M* Transient bactericidal defect of peripheral blood phagocytes from children with acute lymphoblastic leukemia receiving craniospinal irradiation. New Engl. J Med. *289* 1209–1213 1973

4 *Cooper M R DeChatelet L R LaVia M D McCall C E Spurr C L & Baehner R L* Complete deficiency of leukocyte glucose-6-phosphate dehydrogenase with defective bactericidal activity J Clin. Invest. *49* 21a, 1970

5 *Hellum K B & Solberg C O* Influence of anticoagulants on the nitroblue tetrazolium test. Scand. J Infect. Dis. *5* 67–70 1973

6 *Holmes B Quie P G Windhorst D B & Good R A* Fatal granulomatous disease of childhood an inborn abnormality of phagocytic function. Lancet i 1225–1228 1966.

7 *Klebanoff S J* Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes Semin. Hematol *12* 117–142 1975

8 *Koch C* Acquired defect in the bactericidal function of neutrophil granulocytes during bacterial infections. Acta path. microbiol. scand Sect. B *82* 439–447 1974

9 *Lace J K., Tan J S & Watanakunakorn C* An appraisal of the nitroblue tetrazolium reduction test Amer J Med *58* 685–694 1975

10 *McCall C E DeChatelet L R Butler R & Brown D* Enhanced phagocytic capacity the biologic basis for the elevated histochemical nitroblue tetrazolium reaction J Clin Invest. *54* 1227–1234 1974

11 *McCall C F DeChatelet L R., Cooper M R. & Shannon C.* Human toxic neutrophils. III. Metabolic characteristics. J Infect. Dis 127 26–33 1973

12. *Nathan D G* NBT reduction by human phagocytes. New Engl. J Med. 290 280–281 1974

13. *Nathan, D G Baehner R. L. & Weaver D K.:* Failure of nitroblue tetrazolium reduction in the phagocytic vacuoles of leukocytes i chronic granulomatous disease. J Clin. Invest. 48 1895–1904 1969

14 *Nydegger U E., Anner R M., Gerebtzoff A Lambert P H & Miescher P A* Polymorphonuclear leucocyte stimulation by immune complexes. Assessment by nitroblue tetrazolium dye reduction. Europ J Immunol. 3 465–470, 1973

15. *Park B. H Fikrig, S M & Smithwick, E. M* Infection and nitroblue tetrazolium reduction by neutrophils. A diagnostic aid. Lancet ii 532 534 1968.

16. *Park B. H & Good R. A.:* NBT test stimulated Lancet ii 616, 1970

17 *Quie P G* Pathology of bactericidal power of neutrophils. Semin. Hematol. 12 143–160, 1975.

18. *Segal A W* Nitroblue-tetrazolium tests. Lancet ii 1248–1252, 1974

19 *Solberg, C O.* Enhanced susceptibility to infection. A new method for the evaluation of neutrophil granulocyte functions. Acta path. microbiol. scand. Sect. B 80 10–18, 1972.

20. *Solberg, C O & Hellum K. B.* Neutrophil granulocyte function in bacterial infections. Lancet ii 727 730, 1972.

21 *Solberg C O Schreiner A H llum, K B. & Hamre E.* Neutrophil granulocyte function in the early diagnosis of acute myelomonocytic and myeloblastic leukaemia. Acta med. scand. 197 147–151 1975

22. *Stossel, T P.* E aluation of opsonic and leukocyte function with a spectrophotometric test in patients with infection and with phagocytic disorders. Blood 42 121 130 1973

23 *Stossel, T P.* Phagocytosis recognition and ingestion. Semin. Hematol. 12 83–115, 1975.

24 *Wintrobe M M.* Clinical hematology p. 264 London 1967

Acta path. microbiol. scand. Sect. C, 85: 10–16, 1977

C1 SUBCOMPONENTS IN ACUTE PNEUMOCOCCAL OTITIS MEDIA IN CHILDREN

ULF JOHNSON, CARL KAMME, ANNA BRITA LAURELL and NILS INGVAR NILSSON

Departments of Medical Microbiology and Otolaryngology, University of Lund, Lund, Sweden

Johnson, U., Kamme, S., Laurell, A. B. & Nilsson, N. I. C1 subcomponents in acute pneumococcal otitis media in children. Acta path. microbiol. scand. Sect. C, 85: 10–16, 1977.

Twenty children with acute pneumococcal otitis media were studied. In 6 children the infection ran a normal course and healed after the first episode and in 14 it relapsed. The serum levels of the immunoglobulins IgG, IgA and IgM were normal in all 20 children. Specific antibodies to pneumococcal polysaccharide were found in all cases, with no differences in the titers between the relapsed cases and those that healed. The complement components were quantitated with electroimmuno assay. C1q proved depressed in 60 per cent of the relapsed cases and in 16 per cent of the healed cases. C1r and C1s were disproportionally high compared with the C1q levels. Furthermore, crossed immunoelectrophoresis revealed abnormal complexes composed of C1r and C1s, and complexes composed of C1r, C1s and C1 IA. These complexes were more pronounced in sera from the children with relapsing otitis media.

Key words: C1 subcomponents; otitis media; pneumococcus.

Ulf Johnson, Institute of Medical Microbiology, Sölvegatan 23, S-223 62 Lund, Sweden.

Received 30.vi.76 Accepted 7.ix.76

Diplococcus pneumoniae is the causative agent of acute otitis media in about 50 per cent of all cases in children (12). Despite treatment with antibiotics the condition frequently recurs within 14 days and is regularly caused by the same pneumococcal strain as that isolated during the first epidose. *Kamme et al.* (12) found that 26 out of 245 cases of acute otitis media caused by *D. pneumoniae* relapsed.

Several studies of the immunological response to pneumococcal infections of the middle ear have been reported. *Sloyer et al.* (26) demonstrated specific pneumococcal antibodies in patients with acute otitis media. They concluded that the presence of antibodies in the middle ear fluid does not prevent the development of otitis media. *Branefors Helander et al.* (4) found normal immunoglobulin levels in children with acute otitis media.

A possibility that must be borne in mind is that pneumococcal opsonization by specific antibodies (Fc-opsonization) is insufficient and that effective opsonization of the microorganism requires the participation of the complement (C3b-opsonization) (10).

In the present investigation 14 selected children with acute pneumococcal otitis media that relapsed and 6 selected children with acute pneumococcal otitis media that ran a normal course were subjected to an immunological investigation. The levels of the im

munoglobulins IgG IgA and IgM were measured in serum, as was the titer of specific pneumococcal antibodies. Determinations were made of the complement components C1q, C1r C1s, C4 C3 C5 factor B, properdin and C1 IA. The C1 subcomponents were further investigated by crossed immunoelectrophoresis.

MATERIAL AND METHODS

Patients

The clinical material consisted of 20 selected children, all below 6 years of age, with acute pneumococcal otitis media. In 14 children the first episode was followed by relapses, while in 6 of the children the otitis ran a normal course. All the children were treated with penicillin V in a daily dose of 50 mg per kg body weight a day for 10 days.

Isolation of Strains

Pneumococci were isolated from middle ear fluid after myringotomy or spontaneous rupture of the tympanic membrane as described previously (13) I a few of the patients, in whom no fluid from the middle ear was available material from the nasopharynx was used for culture being known that good correlation exists between pathogens found in the nasopharynx and in the middle ear (12). Subculture was done on blood agar plates incubated anaerobically

Typing of the Strains

The serotypes were determined with the Quellung reaction of *Neufeld* (19) and with the co-agglutination technique described by *Kronvall* (14) Type specific antisera to pneumococcal polysaccharides were purchased from Statens Seruminstitut, Copenhagen, Denmark.

Blood Specimens

Serum and EDTA-plasma (Na_2EDTA 5 mmol/l) were obtained on admission and 2 to 3 weeks later In some cases serum and plasma were also collected 6 weeks after admission. All samples were centrifuged and frozen within 6 hours of collection and stored in aliquots at −80° C until analysed.

Quantitation of Immunoglobulin Classes

The immunoglobulins IgG IgA and IgM were quantitated with the electroimmuno assay described by *Grubb* (6, 7)

Specific Pneumococcal Antibodies

Titers of specific pneumococcal polysaccharide antibodies were determined with the indirect immunofluorescence technique with the use of FITC labelled anti-human IgG and anti-human IgM antisera (BBL, Division of Becton, Dickinson and Company USA) as described by *Sloyer et al.* (26)

Quantitation of Complement Components

The complement components C1q, C1s, C4 C3 C5 factor B and C1 IA were quantitated with the electroimmuno assay (24) C1 was quantitated with the electroimmuno assay as described by *Sjöholm et al.* (25) Properdin was quantitated with electroimmuno assay with specific antiserum against properdin incorporated in the gel. The electrophoresis was run for 20 h at 4 V/cm in barbital buffer 75 mmol/l, containing calcium lactate 2 mmol/l, pH 8.6.

Conversion of factor B to Bb was studied in EDTA-plasma by immunoelectrophoresis (9)

Complex formation between the C1 subcomponents and the C1 IA was studied with crossed immunoelectrophoresis as described by *Laurell et al.* (15)

RESULTS

Pneumococcal Serotypes

In all the 14 children with relapsing acute pneumococcal otitis media, the relapse was due to the same serotype of pneumococci as isolated in the first episode. The pneumococcal serotypes isolated from the patients are given in Table 1 The serotypes most frequently found were Nrs 6 and 19

TABLE 1 *Pneumococcal Serotypes Isolated on Admission from 20 Children with Acute Pneumococcal Otitis Media*

Pneumococcal serotype	Number of patients
3	1
6	4
9	1
15	1
18	1
19	6
35	1
NT*	2
ND‡	3

Not typable.
‡ Not determined

Immunoglobulin Levels

The immunoglobulin levels in the acute phase samples obtained on admission from the two patient groups were normal for age (8) Also in the follow up samples the levels were largely unchanged except in those from two patients with relapse, in whom the IgG and IgM were moderately increased.

Specific Antibodies to Pneumococci

Specific antibodies to the capsular polysaccharide of the infecting pneumococcus were demonstrated in all the patients The titer varied between the patients, but no significant difference was found between the relapsed and healed cases The lowest titer noted was 1/16 and the highest 1/512 In 4 out of 14 children with relapse and in 2 out of 6 who healed the titer rose significantly between the first and second sample.

Quantitation of the Complement Components

The normal range of the various complement components was that given by *Sjoholm* (24) For properdin the normal range is 57–153 per cent of a standard pool of 100 sera from healthy blood donors.

C3 and C5 levels were within the normal range. In some patients these components fluctuated moderately between the initial and the consecutive samples C4 was normal except in one patient with relapse In that patient the initial C4 value was low but became normal three weeks later

Properdin tended to show lower levels in the patients with relapses compared with those who healed and with normal children of corresponding age In 1 out of 14 patients with relapsing otitis media a significantly low level was found (Fig 1)

Factor B was invariably normal or elevated and conversion of factor B to Bb could not be demonstrated in any case

The C1 subcomponents C1q C1r and C1s showed abnormalities In the acute phase 9 out of 14 patients with relapsing otitis media and 1 out of 6 without relapse had C1q levels below or close to the lower limit of the normal range. In most of the patients C1q was afterwards normal. The C1r and C1s levels varied with each other and in the initial samples they were disproportionally high compared with the corresponding C1q levels, especially in the relapsed cases (Fig 2) In later samples the C1r and C1s levels were normal. No correlation was found between the degree of C1q depression and the titer of specific pneumococcal antibodies

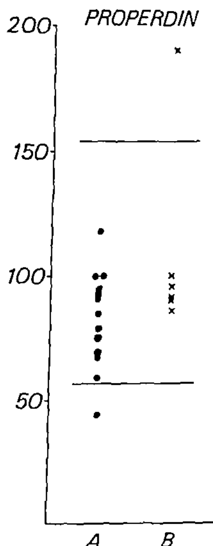


Fig 1 Properdin levels in per cent of a standard pool in acute pneumococcal otitis media A. relapsed cases. B cases without relapse The normal range is indicated.

The C1 IA was elevated in some of the initial samples but usually became normal within three weeks. However in some patients high levels were found in the later samples and then the C1 IA was possibly re-

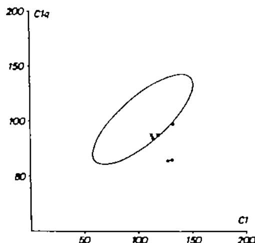


Fig 2 C1q and C1 levels in acute pneumococcal otitis media in per cent of a standard pool. ● denotes relapsed cases and × cases without relapse. The normal ariation of C1q and C1 alues is indicated by 99 per cent confidence ellipse (16)

lated to an abnormal complex formation between C1r and C1s (see below)

Abnormal Complex Formation between C1r and C1s

Crossed immunoelectrophoresis with anti C1s antiserum incorporated in the gel, revealed pronounced complexes of β mobility composed of C1r and C1s and complexes of α_2-mobility composed of C1r C1s and C1 IA (Fig 3 b–d) The nature of these complexes has been investigated by *Laurell et al.* (15) As calcium ions were present during the electrophoretic separation in the first dimension, macromolecular C1 appears at the application slit. In serum from normal individuals, the β-complex is rarely found and in small amounts (Fig 3 a and b) while the α_2-complex is always present in small amounts (Fig 3 a and b)

In the acute phase samples elevated α_2-complexes were found in 9 out of 13 children with relapsing otitis media and in 3 out of 6 children who healed. Excessive amounts of β-complexes were found in all relapsed patients and in 5 out of 6 patients that healed. The amounts of β and α_2-complexes were

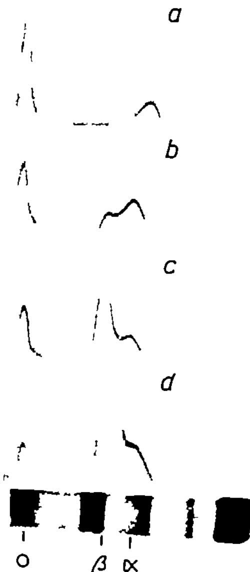


Fig 3 Crossed immunoelectrophoresis with anti-C1 incorporated in the gel showing complexes composed of C1 and C1s (β) and complexes composed of C1 C1s and C1 IA ( ) O denotes the application slit. a, normal serum b c, d serum from children with acute pneumococcal otitis media. The electrophoretic pattern of normal human serum is given for reference. Anode is to the right.

determined by measuring the height of the precipitates. The size was classified into four groups where 0 denotes normal and 1 to 3 increasing amounts of complexes. Fig 4 gives

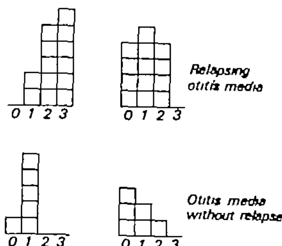


Fig 4 The distribution of β_1 and α_2-complexes in children with relapsing otitis media and with otitis media without relapse. ○ denotes normal and 1 to 3 increasing amounts of complexes.

the amount of complexes found in the two groups. The size of the complexes seemed to correlate with the severity of the infection but not with the amount of specific poly saccharide antibodies found. However the number of patients studied is too small to permit definite conclusions.

DISCUSSION

The serotypes of pneumococci isolated in the material (Table 1) are in good agreement with earlier investigations (13) The serotypes differ from those found in adults (18) and the reason for this is poorly understood

As in earlier studies (4) the immunoglobulin levels were largely normal. The IgG subgroups IgG1 and IgG2 contain the bulk of antibodies with specificity to polysaccharide antigens (11) The finding of specific antibodies to pneumococcal polysaccharide antigens in all the patients does not support the view that defects of the IgG subclasses were the background of relapsing otitis media

A possibility that should be borne in mind is that specific antibodies may not produce sufficient opsonization of the pneumococcus (Fc-opsonization) *Sloyer et al* (26) showed that the presence of type specific antibodies in the middle ear fluid did not prevent development of acute pneumococcal otitis media We did not study the antibodies in such fluid, but despite type specific antibodies in serum acute pneumococcal otitis media relapsed.

Opsonization is enhanced on activation of the complement when C3b is attached to an immune complex (23) *Winkelstein et al.* (28) have shown that C3 plays an important role in the opsonization of pneumococci and thereby in the host defence to pneumococcal infections. This is emphasized by the observation that patients with hypercatabolism of C3 (1) and patients deficient in C3 (2) have severe recurrent bacterial infections, including several of pneumococcal origin.

There is further evidence that the alternate pathway of complement activation may participate in the opsonization and phagocytosis of pneumococci. *Pillemer et al* (20) reported activation of the properdin system upon incubation with pneumococcal polysaccharides. *Ward et al.* (27) found that filtrates from pneumococcal cultures can induce complement mediated chemotactic activity In a recent study *Fine* (5) found differences between pneumococcal serotypes in their ability to activate complement by the alternate pathway Thus, serotypes 7 12 14 and 25 activated the alternate pathway without participation of specific antibodies and serotypes 3 4 and 8 did so only in the presence of type specific antibodies. Pneumococci of serotype 1 were not able to activate the alternate pathway They gave no data on the pneumococcal serotypes 6 and 19 the most common serotypes in our material.

Reed et al. (21) determined the levels of C1q C4 and C3 and factor B in adult patients with pneumococcal pneumonia. They found unchanged serum levels of C1q and C4 in samples obtained on admission and after recovery Factor B was initially depressed but increased on recovery as did C3 However factor B and C3 are known to be acute phase reactants (22, 3) In the present investigation factor B was normal or slightly increased in the acute phase. During the

course of the infection the values increased, as might be expected of an acute phase reactant.

The properdin levels tended to be lower in the group of children that relapsed than in the group of healed children and in healthy children of the same age, which might reflect a consumption of this factor.

The most striking finding in this investigation was the depressed C1q levels and the high levels of C1r and C1s. Complexes containing C1r and C1s protein (25) were found in all children investigated. These complexes are rarely found in normals. Elevated levels of α_2-complexes composed of C1r C1s and C1 IA (25) were found in 70 per cent of the relapsed cases and in 50 per cent of the children in which the disease ran a normal course. The amounts of β and α_2-complexes found in pneumococcal otitis media, especially in the relapsed cases (Fig 2) were far more pronounced than those observed in other diseases reported by *Laurell et al.* (16). The disproportion between the levels of C1q, on one hand, and of C1r and C1s on the other may explain the appearance of these complexes. The number of patients investigated was, however too small to permit evaluation of a prognostic importance of the dissociation of the C1 subcomponents and of the abnormal complexes found.

The reason why the C1q values found were low is unclear. There might be a consumption of C1q due to the presence of immune complexes, but the degree of C1q depression did not vary with the presence or absence of specific antibodies. Furthermore, this explanation will hardly elucidate the disproportion between the levels of C1q on one hand, and the C1r and C1s on the other. It is known that lipid A from endotoxin (17) can interact with C1q. As far as we know no report exists on pneumococcal polysaccharides and C1q in this context.

This work was supported by grants from the *Swedish Medical Research Council* (B77 16X 13C) from *Alfred Österlunds Stiftelse* the *Forssman's Foundation*, the *Medical Faculty University of Lund* and *Malmöhus Läns Landsting*.

REFERENCES

1. *Alper C. A., Block, K. J & Rosen F. S.*. Increased susceptibility to infection in a patient with type II essential hypercatabolism of C3 New Engl. J Med. *288* 601–606, 1973
2. *Alper C A., Colten, H R Rosen F S., Rabson A R., Macnab G. M & Gear J S S.*. Homozygous deficiency of C3 in a patient with repeated infections. Lancet *ii* 1179–1181 1972.
3. *Aronsen K.-F., Ekelund G., Kindmark, C.-O & Laurell, C.-B.*. Sequential changes of plasma proteins after surgical trauma. Scand. J Clin. Lab. Invest. 29 suppl. 124 127–136 1972.
4. *Branefors-Helander P., Dahlberg, T & Nylén O.*. Acute otitis media. A clinical, bacteriological and serological study of children with frequent episodes of acute otitis media. Acta otolaryng. (Stockh.) In press.
5. *Fine D P.*: Pneumococcal type-associated variability in alternate complement pathway activation. Infect. Immun. *12* 772–778, 1975
6. *Grubb A.*: Quantitation of immunoglobulin G by electrophoresis in agarose gels containing antibodies. Scand. J Clin. Lab. Invest. 26 249–255, 1970
7. *Grubb A.* Crossed immunoelectrophoresis and electroimmuno assay of IgM. J Immunol. *112* 1420–1425 1974
8. *Johansson S G O & Berg, T.*. Immunoglobulin levels in healthy children. Acta Paediat. Scand. 56 572 579 1967
9. *Johnson U & Laurell A.-B.*. Complement activation in meningococcal septicemia. Acta path. microbiol. scand. Sect. C, *83* 285–288, 1975.
10. *Johnston J R B Klempner M R., Alper C A & Rosen F S.*. The enhancement of bacterial phagocytosis by serum. The role of complement components and two cofactors. J Exp. Med. *129* 1275–1290, 1969
11. *Kabat E. A.* Heterogenity of antibody to antigenic determinants of polysaccharides. I *Killander J* (Ed.) Nobel Symposium, 3 Gamma Globulins. Almqvist & Wiksell, Stockholm 1967 p. 207–220.
12. *Kamme C., Lundgren K & Mårdh P A.*: The aetiology of acute otitis media in children. Scand. J Infect. Dis. *3* 217–223 1971
13. *Kamme C., Ageberg M & Lundgren, K* Distribution of *Diplococcus pneumoniae* types in acute otitis media in children and influence of the types on the clinical course in penicillin therapy Scand. J Infect. Dis. *2* 183–190 1970.
14. *Kronvall, G* A rapid slide-agglutination method for typing pneumococci by means of specific antibody adsorbed to protein A-con-

taining staphylococci J Med. Microbiol 6 187–190 1973

Laurell A B Mårtensson U & Sjöholm A G C1 subcomponent complexes in normal and pathological sera studied by crossed immunoelectrophoresis. Acta path. microbiol scand Sect C *84* 455–464 1976

Laurell, A B Mårtensson U & Sjöholm A G Studies of C1 subcomponents in chronic urticaria and angio-edema. Sent for publication in Int. Archs Allergy appl Immunol.

Loos M Butler-Suermann D & Dierich M Interaction of the first (C1) the second (C2) and the fourth (C4) component of complement with different preparations of bacterial lipopolysaccharides and with lipid A J Immunol *112* 935–940 1974

Lund E Distribution of age and sex in cases of pneumococcal meningitis. Acta path. microbiol scand. *61* 478–490 1964

Neufeld F Über die Agglutination der Pneumococcen und über die Theorien der Agglutination. Z Hyg Infektkr *40* 54–72, 1902

Pillemer L. Schoenberg M Blum L. & Wurz L. Properdin system and immunity II Interaction of the properdin system with polysaccharides Science *122* 545–549 1955

Reed W P Davidson M S & Williams Jr R. C Complement system in pneumococcal infections. Infect. Immun. *13* 1120–1125 1976

22 *Schutte M., Di Camelli R., Murphy P Sadove M & Gewurz H.* C3 proactivator (C3PA) as an acute phase reactant. Clin. Exp Immunol. *18* 251–256, 1974

23 *Shin H S., Smith M R & Wood W B* Heat labile opsonins to pneumococcus. II Involvement of C3 and C5 J Exp. Med. *130* 1229–1241 1969

24 *Sjöholm A G.* Complement components in normal serum and plasma quantitated by electroimmuno assay Scand. J Immunol. *4* 25–30 1975

25 *Sjöholm A G Mårtensson, U & Laurell, A B* C1r levels in normal human sera determined by electroimmunoassay Acta path. microbiol. scand. Sect. C, *84* 425–427 1976.

26 *Sloyer J L. Howie V M Ploussard J H Ammann A J Austrian R & Johnston Jr R B.* Immune response to acute otitis media in children. I Serotypes isolated and serum and middle ear fluid antibody in pneumococcal otitis media. Infect. Immun. *9* 1028–1032, 1974

27 *Ward P A Lepow I H & Newman L J* Bacterial factors chemotactic for polymorphonuclear leucocytes. Am. J Pathol. *5* 725–736, 1968.

28. *Winkelstein, J A., Smith M R & Shin H S* The role of C3 as an opsonin in the early stages of infection. Proc. Soc. Exp. Biol. Med. *149* 397 401 1975

Acta path. microbiol. scand. Sect. C, 85 17–20 1977

A SIMPLE PROCEDURE FOR DETERMINATION OF BACTERIOLYTIC ACTIVITY IN BIOLOGICAL FLUIDS

DAG BRATLID

Department of Microbiology Institute of Medical Biology and Department of Pediatrics, Institute of Clinical Medicine, University of Tromsø, Tromsø Norway

Bratlid, D. A simple procedure for determination of bacteriolytic activity in biological fluids. Acta path. microbiol. scand. Sect. C, 85 17 20, 1977

A new procedure for spectrophotometric determination of bacteriolytic activity in biological fluids is described. The method uses *Micrococcus lysodeikticus* cells as substrate. By inactivation of the sample through pre-incubation in ice-water a large number of samples can be prepared and analysed simultaneously The necessity of performing the whole analysis in the photometer for one sample at the time is thus eliminated. By measuring the increase in transmission at 570 nm after incubation of the samples at 37° C relatively long reaction time and wide concentration range is obtained. This makes the results quite precise and reproducible. The method has been used to determine the bacteriolytic activity in serum of healthy adults. Men have significantly higher levels than women.

Key words Bacteriolytic activity lysozyme serum spectrophotometry

Dag Bratlid, Department of Pediatrics, University Hospital, University of Tromsø N 9012 Tromsø Norway

Received 2.vii.76 Accepted 19 xii.76

Bacteriolytic activity can be measured in several biological fluids both under normal and diseased conditions (1 2, 5, 6) This activity is thought to be caused mainly by the hydrolytic enzyme lysozyme which has been shown to cause bacteriolysis by rupturing the bond between N-acetyl-glucosamine and N-acetyl-muramnic acid of the bacterial cell wall (4)

Different methods for determining such bacteriolytic activity have been proposed (3 5 7 8) The marked sensitivity of *Micrococcus lysodeikticus* to the hydrolytic activity of lysozyme has led to the use of this organism as a substrate for the bacteriolytic activity

The routine methods presently in use are mainly based on two different technical principles. In the agar diffusion method (5) killed *M lysodeikticus* cells are suspended in agar and poured into a petri dish. Samples to be analysed are deposited in wells cut in the agar and after incubation the clearing zones around the wells are measured. This method is valid over a wide range of concentrations. The agar plates must, however be incubated for several hours before they can be read. In addition, the semilogarithmic relationship between the diameter of the clearing zone and the lysozyme concentration makes the sensitivity of the method dependent upon the concentration of lysozyme.

The turbidimetric assay method is based

TABLE 1 *Light Transmission of Suspensions of Different Concentrations of M lysodeikticus*

Concentration of *M lysodeikticus* mg/ml	Per cent light transmission 400 nm	450 nm	500 nm	550 nm	570 nm	600 nm
5 0	0	0	0	0	0	0
2.5	0	0	0	0 1	0 1	0.1
1 0	0 4	0 4	0.5	0.6	0.6	0 6
0.8	0.5	0.5	0.8	0 8	0.9	1.0
0 6	0.8	0 8	1 0	1 2	1.5	1.8
0.5	1 0	1 0	1.3	1.9	2.2	3.0
0 4	1 2	1.5	2 2	3.5	4 0	5.2
0.2	5 0	7 8	11 8	16.0	18.5	21.0
0 1	19.5	26.5	33 2	40 0	42.5	46.2
0.05	45 0	52.0	58.0	63.5	66.0	68.9
0 01	86.0	89 0	91 0	92 0	93 0	94 0

Suspensions in EDTA phosphate buffer pH 6.2

on spectrophotometric measurement of the clearing of a turbid suspension of *M lysodeikticus* (3 7, 8) In this method the samples are usually added to a suspension of *M lysodeikticus* already pipetted into a cuvette and placed in a spectrophotometer The initial decrease in turbidity (absorption) is then measured, usually within one minute. Using this method only one sample can be analysed at the time and the concentration range is very limited

In the present report a new procedure for the spectrophotometric assay of bacteriolytic

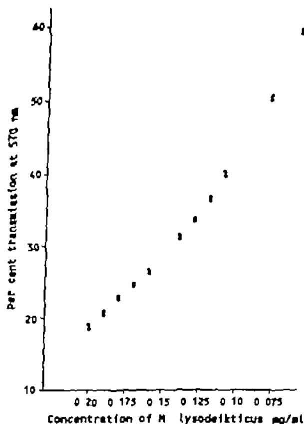


Fig 1 Per cent light transmission at 570 nm of suspensions of different concentrations of *M lysodeikticus* cells in phosphate-EDTA buffer pH 6.2 The results of two different experiments are given.

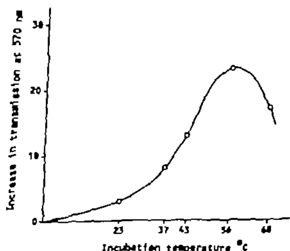
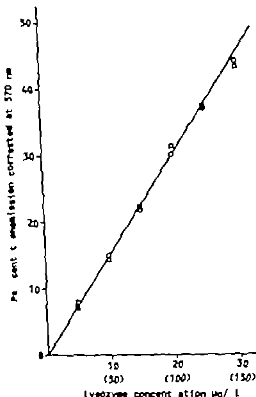


Fig 2 The effect of temperature on the activity of egg-white lysozyme. A 5 0 ml suspension of *M lysodeikticus* cells, 0 * mg/ml, was incubated at different temperatures for 15 minutes with the addition of 0 025 ml of a 10 µg/ml solution of egg-white lysozyme in phosphate buffer pH 7 4



F g 1. Standard curv for the determination of bacteriolytic activity as described under Materials and Methods, using egg-white lysozyme as standard. The points marked □ are the results of experiments using 6 minutes incubation time and correlate with the concentration values in brackets on the abscissa.

activity is described. It is simple to perform, valid over a wide range of concentrations, and several samples can be analysed at the same time The bacteriolytic activity of serum in healthy adults will also be presented.

MATERIALS AND METHODS

Egg-white lysozyme, Koch-Light Laboratories, batch no. 83156 was used as a standard. The enzyme was dissolved in isotonic monopotassium-dsodium phosphate buffer of pH 7.4

Killed, lyophilised *M. lysodeikticus* cells (polynucleotide phosphorylase) Koch-Light Laboratories, batch no. 67485, were used as substrate in the analyses. The bacterial cells were suspended in phosphate-EDTA buffer 0.1 M pH 6.2, prepared by dissolving 10.37 gm of NaH_2PO_4, 7.86 gm of $Na_2HPO_4 \cdot 12H_2O$ and 0.372 gm of EDTA in distilled water to make 1000 ml.

The light transmission of the turbid suspension of *M. lysodeikticus* cells was measured in a Beckman DB GT double beam spectrophotometer. pH levels were determined by a Radiometer pH meter 26

TABLE 2. *Effect of Incubation Temperature on Lysozyme Activity*

Lysozyme conc. µg/ml	Per cent light transmission 1st reading 0° C	2nd reading 37 C	3rd reading 0° C
100	22.0	46.6	48.0
75	21.0	39.5	40.5
50	20.0	32.0	32.8
25	19.0	24.5	25.0
10	18.0	20.5	20.5
5	18.0	19.5	19.5
0	18.0	18.0	18.0

Samples of *M. lysodeikticus* 0.2 mg/ml, 5 ml, were incubated with 25 µl of different concentrations of egg-white lysozyme. The samples were first incubated in an ice-water bath for 30 min and the light transmission was read in the photometer. The second reading was made after the samples had been incubated in 37 C for 5 min and a third reading was made when the samples had been kept in ice-water for another 30 min.

TABLE 3. *Bacteriolytic Activity in Serum of Healthy Adult Blood Donors**

Normal blood donors	Number of subjects	Serum lysozyme concentration µg/ml range	mean value ±	standard deviation
Total	100	4.3–18.4	8.5 ±	2.7
Men	59	4.3–15.7	9.0 ± §	2.9
Women	41	4.6–18.4	7.8 ± §	2.4

* Determined turbidimetrically as described in the text and expressed as µg/ml of egg-white lysozyme.

§ Bacteriolytic activity in serum is significantly higher in men than in women ($p<0.05$)

Methodological Study

Turbidity of suspension of M. lysodeikticus cells in relation to concentration and wavelength. As seen from Table 1 significant light transmission is not found in suspensions in which the concentration of *M. lysodeikticus* cells is above 1.0 mg/ml. With decreasing concentrations, the transmission of the suspension rapidly increases, and light of

2*

TABLE 1 *Light Transmission of Suspensions of Different Concentrations of M. lysodeikticus*

Concentration of *M lysodeikticus* mg/ml	Per cent light transmission					
	400 nm	450 nm	500 nm	530 nm	570 nm	600 nm
5.0	0	0	0	0	0	0
2.5	0	0	0	0.1	0.1	0.1
1.0	0.4	0.4	0.5	0.6	0.6	0.6
0.8	0.5	0.5	0.8	0.8	0.9	1.0
0.6	0.8	0.8	1.0	1.2	1.5	1.8
0.5	1.0	1.0	1.3	1.9	2.2	3.0
0.4	1.2	1.5	2.2	3.5	4.0	5.2
0.2	5.0	7.8	11.8	16.0	18.5	21.0
0.1	19.5	26.3	33.2	40.0	42.5	46.2
0.05	43.0	52.0	58.0	63.5	66.0	68.9
0.01	86.0	89.0	91.0	92.0	93.0	94.0

Suspensions in EDTA-phosphate buffer pH 6.2.

on spectrophotometric measurement of the clearing of a turbid suspension of *M lysodeikticus* (3 7 8) In this method, the samples are usually added to a suspension of *M lysodeikticus* already pipetted into a cuvette and placed in a spectrophotometer The initial decrease in turbidity (absorption) is then measured usually within one minute. Using this method only one sample can be analyzed at the time and the concentration range is very limited

In the present report a new procedure for the spectrophotometric assay of bacteriolytic

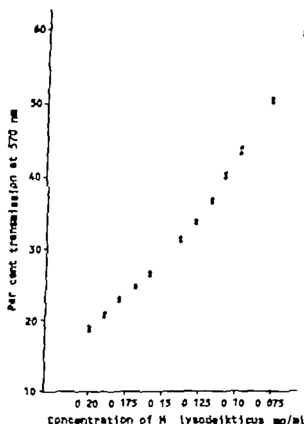


Fig 1 Per cent light transmission at 570 nm of suspensions of different concentrations of *M lysodeikticus* cells in phosphate-EDTA buffer pH 6.2. The results of two different experiments are given.

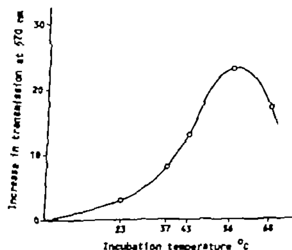


Fig 2 The effect of temperature on the activity of egg-white lysozyme A 5.0 ml suspension of *M lysodeikticus* cells, 0.2 mg/ml, was incubated at different temperatures for 15 minutes with the addition of 0.025 ml of a 10 µg/ml solution of egg-white lysozyme in phosphate buffer pH 7.4

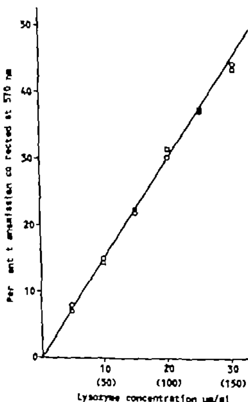


Fig 3 Standard curve for the determination of bacteriolytic activity as described under Materials and Methods, using egg-white lysozyme as standard. The points marked □ are the results of experiments using 6 minutes incubation time and correlate with the concentration values in brackets on the abscissa.

activity is described. It is simple to perform, valid over a wide range of concentrations, and several samples can be analysed at the same time. The bacteriolytic activity of serum in healthy adults will also be presented.

MATERIALS AND METHODS

Egg-white lysozyme Koch-Light Laboratories, batch no. 65156 was used as a standard. The enzyme was dissolved in isotonic monopotassium-disodium phosphate buffer of pH 7.4

Killed, lyophilised *M lysodeikticus* cells (polynucleotide phosphorylase) Koch-Light Laboratories, batch no. 67485 were used as substrate in the analyses. The bacterial cells were suspended in phosphate-EDTA buffer 0.1 M, pH 6.2, prepared by dissolving 10.37 gm of NaH_2PO_4, 7.86 gm of $Na_2HPO_4 \cdot 12H_2O$ and 0.372 gm of EDTA in distilled water to make 1000 ml.

The light transmission of the turbid suspension of *M lysodeikticus* cells was measured in a Beckman DB GT double beam spectrophotometer pH levels were determined by a Radiometer pH meter 26

TABLE 2. *Effect of Incubation Temperature on Lysozyme Activity*

Lysozyme conc. µg/ml	Per cent light transmission		
	1st reading 0° C	2nd reading 37 C	3rd reading 0 C
100	22 0	46 6	48.0
75	21.0	39.5	40.5
50	20.0	32.0	32.8
25	19 0	24.5	25 0
10	18 0	20.5	20.5
5	18.0	19 5	19.5
0	18.0	18.0	18 0

Samples of *M lysodeikticus* 0.2 mg/ml, 5 ml, were incubated with 25 µl of different concentrations of egg-white lysozyme. The samples were first incubated in an ice-water bath for 30 min and the light transmission was read in the photometer. The second reading was made after the samples had been incubated in 37 C for 5 min and a third reading was made when the samples had been kept in ice-water for another 30 min.

TABLE 3 *Bacteriolytic Activity in Serum of Healthy Adult Blood Donors**

Normal blood donors	Number of subjects	Serum lysozyme concentration µg/ml		
		range	mean values ±	standard deviation
Total	100	4.2–18.4	8.5 ±	2.7
Men	59	4.2–15.7	9.0 ± §	2.9
Women	41	4.6–18.4	7.8 ± §	2.4

* Determined turbidimetrically as described in the text and expressed as µg/ml of egg-white lysozyme.

§ Bacteriolytic activity in serum is significantly higher in men than in women ($p < 0.05$)

Methodological Study

Turbidity of a suspension of** M. lysodeikticus **cells in relation to concentration and wavelength. As seen from Table 1 significant light transmission is not found in suspensions in which the concentration of *M lysodeikticus* cells is above 1.0 mg/ml. With decreasing concentrations, the transmission of the suspension rapidly increases and light of

TABLE 1 *Light Transmission of Suspensions of Different Concentrations of M. lysodeikticus*

Concentration of *M lysodeikticus* mg/ml	Per cent light transmission 400 nm	450 nm	500 nm	550 nm	570 nm	600 nm
5 0	0	0	0	0	0	0
2.5	0	0	0	0.1	0.1	0.1
1 0	0 4	0 4	0.5	0.6	0 6	0.6
0 8	0.5	0.5	0.8	0.8	0.9	1.0
0 6	0 8	0.8	1 0	1.2	1.5	1.8
0.5	1 0	1 0	1.3	1.9	2.2	3.0
0 4	1.2	1.5	2.2	3.5	4 0	5.2
0.2	5 0	7 8	11.8	16.0	18.5	21.0
0 1	19.5	26.3	33 2	40 0	42.5	46.2
0 05	43.0	52.0	58 0	63.5	66.0	68.9
0.01	86 0	89 0	91 0	92.0	93 0	94 0

Suspensions in EDTA phosphate buffer pH 6.2.

on spectrophotometric measurement of the clearing of a turbid suspension of *M lysodeikticus* (3 7 8) In this method the samples are usually added to a suspension of *M lysodeikticus* already pipetted into a cuvette and placed in a spectrophotometer The initial decrease in turbidity (absorption) is then measured usually within one minute. Using this method, only one sample can be analysed at the time and the concentration range is very limited.

In the present report a new procedure for the spectrophotometric assay of bacteriolytic

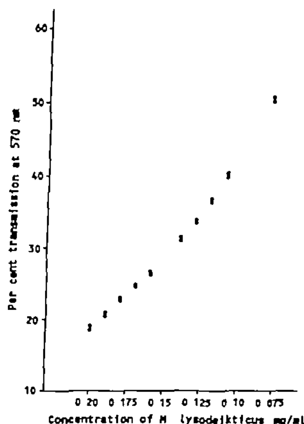


Fig 1 Per cent light transmission at 570 nm of suspensions of different concentrations of *M lysodeikticus* cells in phosphate-EDTA buffer pH 6.2 The results of two different experiments are given.

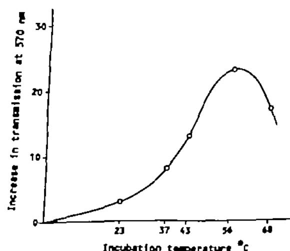


Fig 2 The effect of temperature on the activity of egg-white lysozyme. A 5 0 ml suspension of *M lysodeikticus* cells, 0.2 mg/ml was incubated at different temperatures for 15 minutes with the addition of 0 025 ml of a 10 µg/ml solution of egg white lysozyme in phosphate buffer pH 7 4

Acta path. microbiol. scand. Sect. C, 85 21 25 1977

BACTERIOLYTIC ACTIVITY OF NORMAL AND PATHOLOGICAL CEREBROSPINAL FLUID

DAG BRATLID and KJELL BØVRE

Departments of Pediatrics and Microbiology University Hospital and
Institutes of Clinical Medicine and Medical Biology University of Tromsø Tromsø, Norway

Bratlid, D & Bøvre K. Bacteriolytic activity of normal and pathological cerebrospinal fluid. Acta path. microbiol. scand. Sect. C, 85 21 25, 1977

The bacteriolytic activity of 153 samples of cerebrospinal fluid (CSF) from patients with various diseases was measured by determining the ability of the CSF to cause lysis of a suspension of killed cells of *Micrococcus lysodeikticus*. Normal CSF did not show significant bacteriolytic activity. A high activity was found in patients with bacterial meningitis (mainly meningococcal) only to some extent correlated with the protein and cell content of the CSF. Slight elevation of protein and cell content of CSF in patients with diseases other than bacterial meningitis was not accompanied by significant bacteriolytic activity. The CSF from patient with lymfosarcoma, with as much as 2300 cells/mm³ thus was negative. Also the CSF from patients with serous (viral) meningitis was usually negative. Measurement of bacteriolytic activity in CSF may be of diagnostic importance in cases presenting slight elevation of cell number and protein. Further studies of the significance of the bacteriolytic response in meningitis caused by different microorganisms are warranted.

Key words: Bacteriolysis; cerebrospinal fluid; lysozyme; meningitis; serum.

Dag Bratlid, Department of Pediatrics, University Hospital, University of Tromsø, N 9012 Tromsø, Norway

Received 2. ii 76 Accepted 19 viii.76

The hydrolytic enzyme lysozyme (muramidase) has been shown to cause bacteriolysis by rupturing the bond between N-acetyl glucosamine and N-acetyl-muraminic acid of the bacterial cell wall (10). Activity of this enzyme has been found in plants (9), bacteria (2) and in several animal tissues and fluids (11). In the formed elements of blood, lysozyme has been found especially in granulocytes, whereas lymphocytes are virtually devoid of it (6). Lysozyme is contained in the lysosomes and is liberated into the phagocytic vacuoles during phagocytosis (4). A low level of lysozyme activity is normally encountered in serum from healthy subjects (1 13). Increased serum values of lysozyme have been recorded in patients with severe infectious diseases (13) and high activity of lysozyme has also been found in pus (7). Normal cerebrospinal fluid (CSF) seems not to contain lysozyme (7 8).

The effect of lysozyme is dependent on the kind of bacteria exposed, Gram-positive organisms usually being more susceptible than Gram-negative organisms (7). Lysozyme lysis may be markedly enhanced by other substances such as hydrogen peroxide and ascorbic acid (12). In serum, lysozyme is known to cooperate with other bactericidal and bac

higher wavelengths gives the highest transmission. As shown in Fig 1 the increase in transmission at wavelength 570 nm is almost linear at concentrations from 0.2 mg/ml up to 0 125 mg/ml, where a gradually higher increase is found The wavelength 570 nm and the substrate concentration of 0.2 mg/ml were therefore chosen for the assay procedure

Effect of temperature on bacteriolysis As shown in Fig 2, the bacteriolysis of *M lysodeikticus* caused by lysozyme is very temperature-dependent. At 0 C, almost no lysis takes place. It therefore seems that the bacteriolytic activity can be stopped by keeping the samples in ice water This is also illustrated in Table 2.

Assay Procedure

On the basis of the methodological studies the following procedure has been found useful for spectrophotometric determination of bacteriolytic activity in biological fluids A 0.2 mg/ml suspension of *M lysodeikticus* is prepared by suspending the killed bacterial cells in phosphate-EDTA buffer of 0 1 M and pH 6.2. 5.0 ml of this suspension is pipetted into 100 × 16/17 mm glass test tubes and pre-incubated in ice-water bath for 5 minutes. 0 025 ml of the fluid to be analysed is then pipetted into the substrate while still in the ice bath. When all the samples have been pipetted they are rapidly moved to a water bath of 37 C, shaking at 100 rev/min. The samples are then incubated for 30 minutes, as determined by an alarm clock. After incubation the samples are rapidly returned to the ice-water bath, and the transmission values are read successively in the photometer The photometer has previously been adjusted so that the substrate suspension of 0 2 mg/ml gives a transmission of 15 per cent and the buffer blank 100 per cent at 570 nm. This is also controlled during the analysis. From standard curves, the bacteriolytic activity in the different samples can then be calculated (Fig. 3)

RESULTS AND DISCUSSION

The results of determination of serum bacteriolytic activity in 59 normal adult males and 41 normal adult females are shown in Table 3 As seen from the table the mean values in the males are slightly higher than those in the females.

Recent literature indicates that determination of bacteriolytic activity in biological fluids may have diagnostic and prognostic significance in several diseases including infectious diseases (1 2 5 6) Previous investigators have found both the turbidimetric and the agar plate methods for determination of bacteriolytic activity to be very useful. However as mentioned in the introduction, both methods have weaknesses.

The turbidimetric method has the great advantage that the results can be obtained rapidly The present procedure for spectrophotometric assay of bacteriolytic activity seems to have several advantages compared to previous procedures By introducing the ice water bath, a large number of samples can be analysed and prepared simultaneously and by incubating the samples at 37 C and reading them at 570 nm, a relatively long incubation time and concentration range is obtained. This makes the results quite precise and reproducible. The values for bacteriolytic activity in normal serum found by the present method are quite comparable to those given by other authors (2 8) The previously reported difference in bacteriolytic activity of serum from men and women (8) is also confirmed.

REFERENCES

1 *Bratlid D & Bøvre A* Bacteriolytic activity of normal and pathological cerebrospinal fluid Acta path. microbiol. scand. Sect. C, 85 21–25 1977

2 *Falchuk K R Perrotto J L & Isselbacher K J* Serum lysozyme in Crohn's disease and ulcerative colitis. New Engl. J Med. 292 395–397 1975

3 *Gorin G Wang, S F & Papapavlou L* Assay of lysozyme by its lytic action on *M lysodeikticus* cells Anal Biochem. 39 113–127 1971

4 *Jollès P* Recent developments in the study of lysozymes. Angew Chem., Int. Ed. Engl. 3 28–36 1964

5 *Osserman E. F & Lawlor D P* Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. J exp Med. 124 921–952 1966

6 *Pascual R. S Gee J B. L. & Finch S C* Usefulness of serum lysozyme measurement in diagnosis and evaluation of sarcoidosis. New Engl J Med. 289 1074–1076 1973

7 *Smolelis A N & Hartsell S E.* The determination of lysozyme J Bact. 58 731–736 1949

8. *Zucker S Hanes D J., Vogler W R & Eanes R Z* Plasma muramidase A study of methods and clinical applications. J Lab. clin. Med. 75 83–92 1970

TABLE 1 *Bacteriolytic Activity, Cell and Protein Content of CSF from the various Groups of Patients Included in the Study*

No. of samples	Cell content, cells/mm³		Protein content, mg/100 ml		Bacteriolytic activity µg lysozyme/ml	
	Range	Mean	Range	Mean	Range	Mean
Patients with normal CSF						
53	0–8	2	10–48	29	0–1.0	0.0
Pathological CSF without infection						
20	13–2500	204	56–105	70	0–1.0	0.2
Serous (viral) meningitis						
11	11–1000	241	30–92	51	0–2.5	0.4
Bacterial meningitis/meningococcal septicaemia						
68	1–35000	3712	10–1100	240	0–160	22.7

TABLE 2. *Bacteriolytic Activity in Serum or CSF from Patients with Dominantly Meningococcal Meningitis or Meningococcal Septicaemia*

	Bacteriolytic activity µg lysozyme/ml	
	Serum	CSF
Meningitis		
Patient no 1	7.0	55 0
2	7.0	61.5
3	12 0	31.0
4	4.7	31.0
5	4 1	33.0
6	11.0	45.5
7	12.0	68 0
Septicaemia		
Patient no. 8	50.8	3.0
9	6 0	0.0
10	2.5	0.5
11	4.0	0 0
12	4.1	1.0
13	13.3	0.0
14	9.9	0.0
15	18.1	0 0
16	12.8	0.0

disease of the central nervous system showed increased values of protein and/or cell content. Six of these patients had malignant disorders such as leukaemia (four patients) or lymphomas (two patients) others had systemic diseases (four patients) As shown in Table 1 no significant bacteriolytic activity was found in these samples. A patient with lymphosarcoma and as much as 2500 cells/mm³ thus showed no bacteriolytic activity at all.

Serous (viral) meningitis. Eleven patients (age 10–38 years) with viral meningitis, mostly mumps (10 patients) were also examined. This group also almost completely lacked bacteriolytic activity in spite of a substantially elevated cell content (Table 1) The protein content was, however close to normal in this group of patients. The highest cell count was found in a patient with mumps, 1000 cells/mm³ without any measurable bacteriolytic activity

Bacterial meningitis Sixty-eight samples of CSF from patients (age 0–74 years) with bacterial meningitis were studied. In 45 patients the disease was caused by *Neisseria meningitidis* four patients had infection with *Haemophilus influenzae* and in 10 cases the cause of the disease was unknown. Included in this group were also nine patients with meningococcal septicaemia and almost normal CSF As shown in Table 1 a substantial elevation of the bacteriolytic activity was found in most of the samples. Patients with septicaemia usually had normal or low levels of bacteriolytic activity although meningococci were grown from several of the CSF samples. Several patients showed bacteriolytic

teriolytic factors, especially the antibody complement system and a factor named beta lysin (5)

Lysozyme is usually quantitated by the ability of the enzyme to cause lysis of a suspension of *Micrococcus lysodeikticus* (1 7 11, 13) Although the lytic activity of biological fluids measured in this way mainly may be due to lysozyme alone and is assayed in units of lysozyme activity the term bacteriolytic activity is presently preferred leaving open the question whether enhancing mechanisms are involved and the possibility that other lytic factor may be present. In this study the results of determination of bacteriolytic activity in CSF from patients with infectious diseases of the central nervous system as well as other diseases are reported

MATERIALS AND METHODS

CSF from 153 patients with various diseases were examined. The samples were analysed on the day of sampling or kept in refrigerator until they were analysed This delay did not influence the results. The values for protein and cell content of the CSF were obtained from the patient records.

Bacteriolytic activity was measured by the ability of CSF to cause lysis of a suspension of killed cells of *M lysodeikticus* using the turbidimetric method described by *Brathal* (1) Killed lyophilized *M lysodeikticus* cells were obtained from Koch Light Laboratories, batch no 67485 Egg White lysozyme Koch-Light Laboratories, batch no 65156 was used as standard. The values for bacteriolytic activity were given in µg egg white lysozyme/ml of CSF

RESULTS

Normal CSF Fifty three samples of normal CSF from patients (age 0–74 years) were included in the study The samples were mostly taken from patients suffering from various febrile conditions or cerebrovascular disease as part of the routine examination of these patients. As shown in Table 1 no significant bacteriolytic activity was found in these samples, in agreement with the report by *Fleming* (7)

Pathological CSF without infection Twenty one samples of CSF from patients (age 3–87 years) without clinical signs of inflammatory

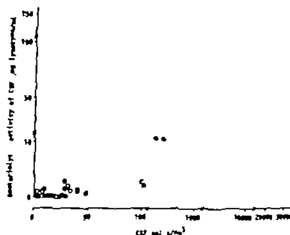


Fig 1 The bacteriolytic activity of CSF from patients with bacterial meningitis correlated with the cell content of the sample.

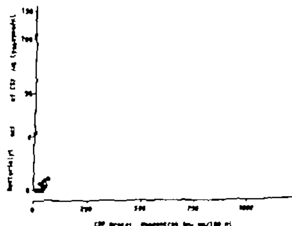


Fig 2 The bacteriolytic activity of CSF from patients with bacterial meningitis correlated with the protein concentration in the sample.

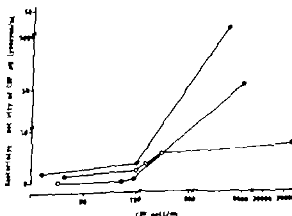


Fig 3 The bacteriolytic activity and cell content of serial CSF samples from three patients treated for bacterial meningitis.

light on the connection between the bacteriolytic activity observed and phagocytosis and possible host-dependent variables of the activity. Besides, it seems warranted to study the elicitation and effect of the bacteriolytic response in relation to endotoxin and the specificity of bacterial strains causing the disease.

REFERENCES

1. *Bratlid, D.:* A simple procedure for determination of bacteriolytic activity in biological fluids. Acta path. microbiol. scand. Sect. C, 85 17–20 1977
2. *Brown W C Wilson, C R., Lukhart S Young F E. & Shiflett M A.:* Antibacterial activity of a lysozyme-like enzyme from staphylococci. Antibiotiki 20 907 911 1975
3. *Chartrand S A. & Cho C T.:* Persistent pleocytosis in meningitis. J Pediat. 88 424–426 1976.
4. *Cohn, Z. A. & Wiener E.* The particulate hydrolases of macrophages. II Biochemical and morphological response to particle ingestion. J exp. Med. 118 1009–1020 1963
5. *Donaldson, D M., Robert R. R., Larsen, H S & Tew J G.:* Interrelationship between serum beta-lysin, lysozyme, and the antibody-complement system in killing *Escherichia coli.* Infect. Immun. 10 657–666, 1974
6. *Flanagan, P & Lionetti, F.:* Lysozyme distribution in blood. Blood 10 497–501 1955
7. *Fleming, A.:* On a remarkable bacteriolytic element found in tissues and secretions. Proc. Roy Soc., Ser B, 93 306–317 1922.
8. *Fleming, A & Allison V D.:* Observations on a bacteriolytic substance ("lysozyme") found in secretions and tissues. Brit. J exp. Path. 3: 252–260 1922.
9. *Howard J B. & Glazer A. N* Papaya lysozyme. Terminal sequences and enzymatic properties. J biol. Chem. 244 1399–1409 1969
10. *Jollès, P.:* Recent developments in the study of lysozymes. Angew Chem., Int. Ed. Engl. 3 28–36 1964
11. *Litwack G.:* Photometric determination of lysozyme activity Proc. Soc. exp. Biol. Med. 89 401–403, 1955.
12. *Miller T E.:* Killing and lysis of gram-negative bacteria through the synergistic effect of hydrogen peroxide, ascorbic acid, and lysozyme. J Bact. 98 949–955 1969
13. *Zucker S., Hanes D J., Vogler W R. & Eanes R. Z.:* Plasma muramidase: A study of methods and clinical applications. J Lab. clin. Med. 75 83–92, 1970.

activity above 100 μg/ml, which is almost 20 times higher than normal serum levels of lysozyme (1, 13). Figs. 1 and 2 indicate a gross correlation between cell and protein content and bacteriolytic activity although many samples do not fit exactly into this pattern. A patient with *H. influenzae* meningitis and as much as 33 000 cells/mm³ thus showed a bacteriolytic activity of only 6.7 μg/ml of lysozyme, whereas a patient with tuberculous meningitis and a cell count of 270 cells/mm³ showed an activity of 10.6 μg/ml. Consecutive samples from three patients were analysed (Fig. 3). Two of these patients showed a parallel lowering both of cell count and bacteriolytic activity during treatment. The third patient showed a rapid loss of bacteriolytic activity in spite of maintenance of a moderately elevated cell count.

Serum samples from 16 patients with meningococcal septicaemia or meningococcal meningitis were also analysed. As shown in Table 2, no correlation between the bacteriolytic activity of serum and CSF was found.

DISCUSSION

The results of the present study indicate that determination of bacteriolytic activity in CSF is of diagnostic significance in patients suspected of inflammatory disease of the central nervous system. Thus, no bacteriolytic activity was found in CSF with normal cell and protein content or in pathological CSF from patients with non-inflammatory disease. It applies in general that the bacteriolytic activity was not significally elevated in patients with serous (viral) meningitis. In patients with bacterial meningitis, however, a marked elevation of bacteriolytic activity in CSF was most often found. Thus, it appeared that there was a gross correlation between cell and protein content and bacteriolytic activity. Since cases of bacterial meningitis with low bacteriolytic activity of CSF also were seen, the main diagnostic importance of this study appears to be that a clearly elevated bacteriolytic activity of CSF strongly supports the diagnosis of bacterial meningitis.

In a recent study (3) the persistent pleocytosis that in spite of treatment and clinical improvement may be found in some patients with meningitis, was discussed. Two of the three patients with bacterial meningitis particularly followed in the present study showed a parallel decrease of bacteriolytic activity and cell count of CSF, whereas the third patient lost the bacteriolytic activity before the cell count had returned to normal. Such a difference may be of relevance whenever the stage at which to discontinue the treatment of patients with persistent pleocytosis has to be decided. Thus, the absence of bacteriolytic activity may indicate that the bacterial disease is no longer active.

In some patients with high cell and protein content of CSF the bacteriolytic activity at the height of disease was found to be much lower than that expected. This may indicate that the phagocytic activity and/or the bacteriolytic response of these patients were lower than normal or that the specific organism did not stimulate these mechanisms.

In some patients with a moderate cell count of CSF a marked bacteriolytic activity was still found. This was particularly distinct in a patient with tuberculous meningitis, and may indicate involvement of the fixed macrophages of the central nervous system.

The complete lack of correlation between bacteriolytic activity of serum and CSF probably indicates that lysozyme does not pass easily across the blood-brain barrier. The high level of bacteriolytic activity of CSF often found in bacterial meningitis, far above the reported values applying to serum in patients with infectious diseases (13), may also indicate that lysozyme (or other bacteriolytical principle) is particularly available and perhaps of special importance for the defence against bacterial infections in the central nervous system. This is in agreement with Fleming & Allison (8) who found a very high lysozyme content in the meningeal cells.

Generally, the results of the present study point to interesting phenomena related to defence and pathogenesis in bacterial meningitis. Further studies are needed to throw

liver disease. Furthermore, the presence of immunoglobulin bearing cells in liver biopsies from patients in the two groups was compared.

PATIENTS AND METHODS

The material comprised 25 consecutive patients with biopsy-verified chronic active hepatitis or non-alcoholic cirrhosis. Twelve had active cirrhosis, 12 chronic aggressive hepatitis and one chronic persistent hepatitis according to the morphological criteria set up by *De Groot et al.* (6). The age ranged from 22 to 77 years (mean 55). 20 of the patients were women and five were men. At the time of investigation, seven patients were treated with corticosteroid and three with azathioprine because they participated in a controlled study.

Seventeen persons (staff members and surgical patients with no sign of liver disease) matched with regard to sex and age served as controls.

HB_sAg and hepatitis B antibody (HB_sAb) were demonstrated by immuno-electro-osmophoresis as described by *Prince & Burke* (17) and Ausria II 125 and Ausab (Abbott Diagnostic Division).

For determination of antinuclear antibodies (ANA) and smooth muscle antibodies (SMA) an indirect immunofluorescence technique was employed, as described previously (3).

Enumeration of T, B and O cells. Lymphocytes were isolated by Hypaque/Ficoll (lymfoprep from Nyegaard & Co., Oslo) gradient separation of heparinized venous blood samples. In the samples studied, some of the isolated cells were monocytes, as determined by phagocytosis of polystyrene particles.

The T cells were identified by E-binding technique (sheep red blood cell technique). I. Hanks Balanced Stock solution, freshly separated lymphocytes and sheep red blood cells stabilized by absorbed AB serum were incubated for 18–20 hours at 4 C. All lymphocytes with three or more adherent sheep erythrocytes were regarded as E-rosettes.

B cells were enumerated using direct immunofluorescent staining with FITC-labelled monospecific rabbit antihuman Ig serum (Dako-Immunoglobuline A/S, Copenhagen) for cells containing surface IgG, IgA and IgM. All mononuclear cells with less than four polystyrene particles in the cytoplasm and with clear membrane fluorescence were included as B cells.

Lymphocytes not identified as T cells or B cells were regarded as null cells. For technical reasons, T-lymphocytes were not calculated in one patient and in two controls. IgA bearing lymphocytes were not determined in three of the controls.

From total lymphocyte counts performed on the same sample of venous blood, the absolute number of T and null cells was calculated in 21 of the patients and in 15 controls, the absolute number of B cells being calculated in 22 patients and in all controls.

Simultaneous determination of serum immunoglobulins was carried out in 21 of the patients by a modification of the Laurell immuno-electrophoresis (21).

Immunohistochemical investigation. In nine of the patients, a liver biopsy was performed according to *Menghini* (13) in relation to the peripheral lymphocyte enumeration. The liver tissue was immediately cut into 3 μ thick slices on a cryostat, dried for one hour using an air blower, treated with phosphate buffer (pH 7.1) for 5 minutes, ethanol (93 per cent) for 2 × 5 minutes, phosphate buffer for 2 × 5 minutes and finally with distilled water for 2 × 5 minutes.

All liver sections were tested by conjugated monospecific rabbit antihuman Ig sera for IgG, IgA and IgM containing cells. The specificity of the fluorescence was confirmed by specific blocking tests.

Sections treated with conjugated antibodies were first incubated with the antibody in a humid atmosphere for 30 minutes at room temperature and then washed quickly under running water. Sections were then placed in distilled water for 2 × 5 minutes, phosphate buffer for 4 × 5 minutes and distilled water for 2 × 5 minutes. The coverglasses were then placed together with a few drops of a 20 per cent glycerol trisbuffer (pH 8.4).

For control, sections were incubated with unconjugated antibodies prior to incubation with conjugated antibody in a humid atmosphere for one hour at 37 C, washed quickly under running water, placed in distilled water for 2 × 2 minutes and then treated as mentioned above under the conjugated antibodies.

The number of immunoglobulin containing cells (IgG, IgA and IgM) were calculated in each biopsy and, on the basis of these figures and the size of the biopsy, a semiquantitative evaluation was performed.

The Mann-Whitney rank sum test was used in the statistical analysis.

RESULTS

In eight patients, HB_sAg was found in serum. In six, the antigen was demonstrated by both techniques, and in two by radio-immuno assay only. Anti-HB_sAg was not detected in any of the patients by immuno-electro-osmophoresis, but eight had positive reaction by radio-immuno assay. HB_sAg and anti-HB_sAg occurred simultaneously in one of the patients. Thus the series can be divided into

Acta path. microbiol scand Sect. C 85 26–32 1977

CIRCULATING T AND B LYMPHOCYTES AND IMMUNOGLOBULIN CONTAINING CELLS IN THE LIVER IN CHRONIC ACTIVE LIVER DISEASE

JAN ALDERSHVILE, ODD DIETRICHSON, FINN HARDT and JENS O NIELSEN

2nd Medical Department, Kommunehospitalet, Division of Hepatology Hvidovre Hospital and Department of Infectious Disease Rigshospitalet, Copenhagen, Denmark

Aldershvile J., Dietrichson, O., Hardt, F & Nielsen, J O Circulating T and B lymphocytes and immunoglobulin containing cells in the liver in chronic active liver disease. Acta path. microbiol scand. Sect. C, 85 26–32 1977

The number of circulating T and B lymphocytes was estimated in 25 patients with biopsy-verified chronic non alcoholic liver disease Fifteen of these had circulating HB_sAg and/or anti-HB Ag and 10 were without these markers of HB virus infection. In both groups of patients a significant decrease of T cells and a parallel significant increase in null cells was found but any difference with respect to T and null cells in patients in the two groups was not observed. Liver biopsies from five of the patients with and four without HB_sAg and/or anti HB Ag were studied for the presence of immunoglobulin bearing cells. In three out of five liver biopsies from the HB Ag and/or Ab positive patients and in two out of the four liver biopsies from the HB Ag and anti HB Ag negative patients, a heavy periportal infiltration with plasma cells was found. However the number and classes of the immunoglobulin containing cells could not be correlated either to the histological evaluation of the stage of activity of the liver disease or to the markers for HB virus infection. The immunological findings in the two groups of patients with chronic liver disease seem to be of the same nature *and are most likely a consequence of the liver disease and not the cause of it.*

Key words T and B lymphocytes immunoglobulin containing cells chronic active liver disease.

J Aldershvile, 2nd Medical Department Kommunehospitalet DK 1399 Copenhagen K, Denmark.

Received 17 vii 76 Accepted 25.x.76

It has recently been suggested that the immunopathological process in chronic active hepatitis in most cases is initiated by a hepatitis B virus infection (10) In hepatitis B antigen (HB_sAg) positive patients, the T cell function is supposed to be normal but the antibody production by B cells is impaired causing a defect in clearing HB virus. In contrast, in the HB_sAg negative group the suppressor effect of T cells on B cell activity is defect. The virus is cleared, but has initiated an auto-immune reaction.

The purpose of the present study was to compare the number of circulating T cells and the number and classes of circulating B cells in peripheral blood in patients with HB_sAg positive and HB_sAg negative chronic

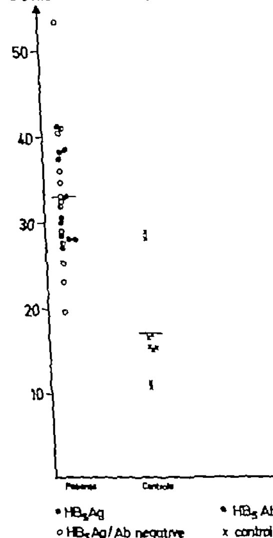


Fig 1 c

percentage of null cells in the patients as compared with controls ($p<0.01$) There was no difference between the HB_sAg/Ab positive and negative group.

Fig 2 (a, b, c) demonstrate the percentage of B cells in different subclasses in 21 of the patients compared with the levels of serum immunoglobulins. No correlation was found

In Fig. 3 the absolute number of T B and null cells is calculated. Again, the number of T cells is seen to decrease and the number of null cells to increase in the patients as compared with findings in the control group, but only the difference in null cells is statistically significant ($p<0.01$)

No difference between patients on immunosuppressive drugs and those without was found.

The immunohistochemical studies were carried out on frozen liver sections from three patients with HB_sAg two patients with anti-HB_sAg, and four patients with neither HB_sAg nor anti-HB_sAg in serum. In the three HB_sAg positive subjects, a heavy infiltration with plasma cells in the periportal areas was found. These cells appeared to have cytoplasmatic fluorescence with an equal number of cells reacting with anti IgM, anti IgG and anti IgA. In two of the four biopsies from HB_sAg/Ab negative patients a high number of immunoglobulin containing cells was also found. The variation in number and classes of immunoglobulin containing cells, however could not be correlated to the histological evaluation of the stage of activity of the liver disease (6)

DISCUSSION

There is evidence suggesting that the aetiological or pathogenical mechanism in HB_sAg positive and HB_sAg negative chronic liver disease may be different (8) Persistence of HB_sAg in acute viral hepatitis indicates continuous viraemia and development of chronic active liver disease (CALD) might occur (14) In HB_sAg negative patients, an altered immune mechanism seems to be of considerable importance for the liver damage. In only a few cases there is evidence for drug induced liver disease (8) or initial type A hepatitis (9)

If HB virus infection is the initiating factor in both HB_sAg positive and HB_sAg negative CALD due to different immunological disturbances as suggested (10 16) marked differences in the immunological functions in the two groups should be expected. Furthermore, the number of patients with circulating

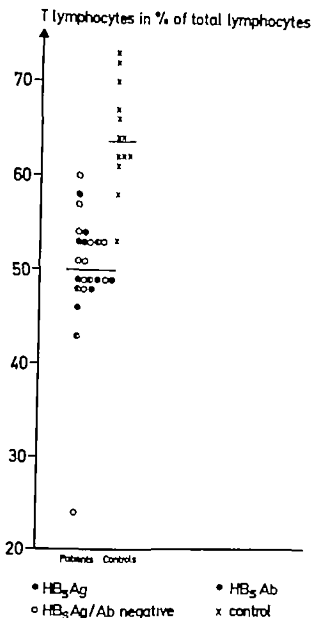


Fig 1 a.

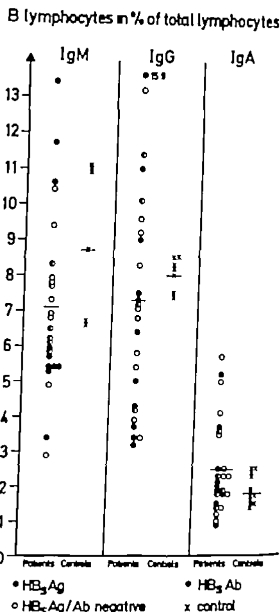


Fig 1 b

Fig 1 a) T lymphocytes, b) subclasses of B lymphocytes, c) null cells in per cent of total number of circulating lymphocytes in patients with chronic liver disease and controls.

two groups, one comprising 15 patients with HB$_s$Ag and/or anti HB$_s$Ag the other comprising 10 patients without these markers.

Smooth muscle antibodies (SMA) and/or antinuclear antibodies (ANA) were present in serum in all the HB$_s$Ag/Ab negative patients, and in six patients from the positive group viz. all patients with anti HB$_s$Ag

As will be seen from Fig 1 a there was a significant decrease in the percentage of T cells in the patients as compared with the control group ($p<0.01$) The percentage of T cells in HB$_s$Ag/Ab positive patients did not differ from that of the negative group

In Fig 1 b the distribution of B cells in percentage of the total lymphocytes is demonstrated No significant difference between the patients and the controls was found or between patients with and without HB$_s$Ag/Ab in serum with respect to total number and subclasses.

Fig 1 c shows a significant increase in the

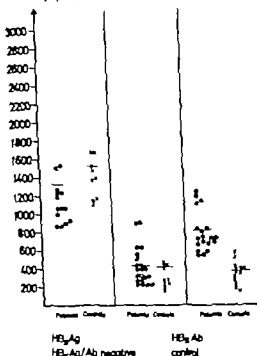


Fig 3 Absolute number of T and B lymphocytes and null cells in patients with chronic liver disease and controls.

phocytes. The existence of a rosette inhibiting factor which might "hide" some of the T lymphocytes has also been postulated and that careful washing could remove this factor (4) Finally the increased number of null cells could be a reality indicating the presence of killer cells (K cells) with active cytotoxicity directed against hepatocytes in patients with CALD (5)

Our finding of an increased IgG in the HB_sAg/Ab negative group is in agreement with previous observations (8, 20) and, like *Vittal et al* (20) we found no difference in IgA level. If compared with findings in the patients described by *Vittal et al.*, normal IgM could be due to a less active liver disease.

Any correlation between the level of the different classes of immunoglobulins and the corresponding number of circulating B cells was not found.

In five of the nine liver biopsies studied, a heavy infiltration with immunoglobulin containing cells was found, and equal distribution within the subclasses (IgG IgA and IgM) This is in contrast with findings by others (11 12) who have reported dominance of IgG and IgA containing mononuclear cells in CALD The immunofluorescence studies have not revealed any difference between HB_sAg negative and HB_sAg positive CALD

The immunological changes observed in the present study in two groups of patients with CALD seem to be of one and the same nature and are most likely a consequence of the liver disease and not the cause of it.

The work was supported by grants from the *Danish Medical Research Council* (512/1960-72, 512/3077 73 512/4019 + 5727 75) *Ebba Celinder's Foundation*, and our thanks are due to *Anne Marie Møller* for valuable laboratory assistance.

REFERENCES

1 *Barker L. F., Peterson M. R., Shulman, N. R. & Murray R.:* Antibody responses in viral hepatitis, type B. J amer med. Ass. *223:* 1005–1008, 1973
2 *Bernstein I M., Webster K. H., Williams R. C & Strickland R. G.:* Reduction in circulating T lymphocytes in alcoholic liver disease. Lancet *ii:* 488–490, 1974
3. *Christoffersen P., Dietrichson O., Faber V & Poulsen, H* The occurrence and significance of abnormal bile duct epithelium in chronic aggressive hepatitis. Acta path. microbiol. scand. Sect. A, *80* 294–302, 1972.
4 *Chisari, F V & Edgington, T S.*. Two mechanisms of null cell generation in a prototype human viral infection. Fed. Proc. *34* 1012, 1975
5. *Cochrane A. M G., Moussouros A., Thomson A. D., Eddleston, A L. W F & Williams, R.:* Antibody-dependent cell-mediated (K cell) cytotoxicity against isolated hepatocytes in chronic active hepatitis. Lancet *i* 441–444 1976.
6. *De Groote J Desmet V J Gedigk P., Korb G Popper H., Poulsen H., Scheuer P J., Schmid M Thaler H., Uehlinger E. & Wepler W A.* Classification of chronic hepatitis. Lancet *ii* 626–628, 1968.
7 *De Horatius, R. J Strickland R. G. & Willi-*

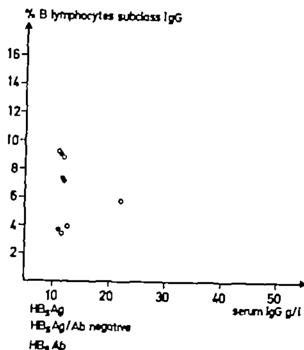


Fig 2 a

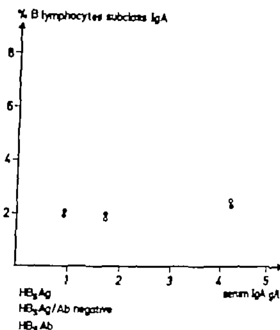


Fig 2 b

Fig 2 B lymphocytes a) subclass IgG b) subclass IgA, c) subclass IgM in per cent of total number of circulating lymphocytes compared with simultaneous determinations of serum immunoglobulins in patients with chronic liver disease.

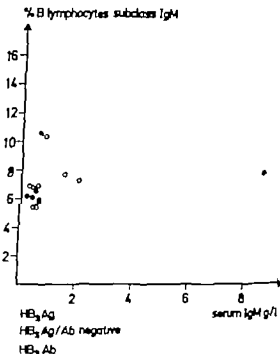


Fig 2 c

antibody to HB$_s$Ag should be found to be higher in the HB$_s$Ag negative group

Barker et al (1) have demonstrated persistence of anti HB$_s$Ag for years in at least 80 per cent of individuals with previous HB$_s$Ag positive hepatitis. It is therefore unlikely that the HB Ag/Ab negative and all the Ab positive CALD among patients in our study are derived from patients with acute viral hepatitis type B Furthermore, the presence of the specific antibody to HB$_s$Ag seems to have no influence on the development of CALD (1)

In our study the number of circulating T lymphocytes was reduced to the same degree in Ag/Ab positive and Ag negative patients. *This is in agreement with the study carried* out by *De Horatius et al.* (7)

A reduction in circulating T cells has been observed in a variety of chronic diseases (ulcerative colitis, sarcoidosis, arthritis rheumatoides, chronic alcohol abuse, etc.) (2 18) and seems not always to have an *influence on* the functional capacity of the cellular immune system and thus, immune response to skin tests and *in vitro* tests may be found to be normal in patients with CALD (15 19)

The *increased number of null cells in the* patients could be due to insufficient sensitivity of analysis and the genuine picture might be *qualitative changes in* T and/or B lym-

Acta path. microbiol. scand. Sect. C, 85 33–40, 1977

IMMUNE RESPONSE IN MICE TO HAPTEN CONJUGATED SEPHAROSE

SAIJA KOSKIMIES

Department of Serology and Bacteriology University of Helsinki,
Haartmaninkatu 3 00290 Helsinki 29 Finland

Koskimies, S. Immune response in mice to hapten conjugated Sepharose. Acta path. microbiol. scand. Sect. C, 85 33–40 1977

Mice were injected intraperitoneally with Sepharose 4B beads coupled with hapten NIP and their anti-NIP response was studied by counting antibody forming cells and determining serum titers. Mice responded well to doses of 0.7 ml of packed beads but 0.3 and 1.2 doses induced much weaker responses. Anti-NIP titers in recipients of 0.7 ml of the antigen lasted nearly constant for at least 7 weeks. Both T cell status of the recipient and use of adjuvant had an effect on the response. Antigen without adjuvant induced primarily IgM antibodies in normal mice, but IgM and IgG in nude mice. When *Hemophilus pertussis* or polyacrylic acid was used as adjuvant both normal and nude mice produced IgM and IgG antibodies, and normal mice produced in addition IgA antibodies.

Key words Immune response hapten conjugated Sepharose mice.

S Koskimies, Department of Serology and Bacteriology University of Helsinki Haartmaninkatu 3, 00290 Helsinki 29 Finland.

Received 26. iii.76 Accepted 26.viii 76

Immunogenicity of a hapten directly coupled onto an inert carrier has been studied in cultures of lymphoid cells (13, 17) *Katz & Unanue* (17) found that DNP coupled onto the culture dish did not induce an anti-DNP response but *Feldmann et al.* (13) could get a good response in spleen cell cultures with TNP-polyacrylamide beads. In this study haptenated non-digestable beads have used for immunization of whole animals, mice. Such studies are interesting because of the old postulate that the antigen has to be digestable by hydrolytic enzymes (4) Another reason making such beads interesting immunogens is the fact that they adsorb considerable amounts of antibody Also, because of their physical uniqueness they may induce different immunoglobulins than conventional antigens. Finally the effect of T cell deprivation on such immunization was considered worth of a study

MATERIALS AND METHODS

Antigens. The hapten used in all experiments was 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) coupled to various carrier molecules.

Hapten molecules were conjugated to agarose (Sepharose) using the method of *Cuatrecasas* (6) Sepharose 4B (Pharmacia, Uppsala) was activated with cyanogen bromide, 50 mg per ml of Sepharose. After washing on a sintered glass filter with cold 0.1 M sodiumbicarbonate NIP-ethylendiamine was added in ratio 1.5 mg per 1 ml of packed Sepharose and the mixture was kept at 4 C under constant stirring for 16 h. The NIP-Sepharose conjugate (NIP Seph) was washed several times with borate buffer pH 8.5 8M urea, citrate buffer pH 2.6 and carbonate buffer pH 10 in this order Residual activity was inactivated with 0.05M

ams R C T and B lymphocytes in acute and chronic hepatitis. Clin. Immunol. Immunopath *2* 353–360 1974

8. *Dietrichson O* Chronic active hepatitis. Scand. J Gastroent. *10* 617–624 1975
9. *Dietrichson O Juhl E Christoffersen P Elling P., Faber V Iversen K Nielsen J O Petersen P & Poulsen H* Acute viral hepatitis Factors possibly predicting chronic liver disease. Acta path. microbiol. scand. Sect A *83* 183–188 1975
10. *Eddleston A L W F & Williams R.* Inadequate antibody response to HB_sAg or suppressor T-cell defect in development of active chronic hepatitis. Lancet *ii* 1543–1545 1974
11. *Hadziyannis S Feizi T., Scheuer P J & Sherlock S* Immunoglobulin-containing cells in the liver Clin. exp Immunol *5* 499–514 1969
12. *Husby G Blomhoff J P Skrede S Elgjo K & Gjone E* Detection of immunoglobulins in paraffin-embedded liver biopsies. Scand J Gastroent. *8* 621–629 1973
13. *Menghini G* One-second needle biopsy of the liver Gastroenterology *35* 190–199 1958.
14. *Nielsen J O Dietrichson O Elling P & Christoffersen P* Incidence and meaning of persistence of Australia antigen in patients with acute viral hepatitis. New Engl. J Med. *285* 1157–1160 1971
15. *Nielsen J O Reinicke V., Dietrichson O., Andersen V Thomsen M & Andersen E.* Immunological studies of Australia antigen in carriers with and without liver disease. Clin exp Immunol. *15* 9–16 1973
16. *Popper H & Mackay I R* Relation between Australia antigen and autoimmune hepatitis. Lancet *i* 1161–1163 1972
17. *Prince A M & Burke K* Serum hepatitis antigen (SH) Rapid detection by high voltage immunoelectroosmophoresis. Science *169* 593–595 1970.
18. *Strickland R G Korsmeyer S Soltis R D., Wilson I D & Williams R C* Peripheral blood T and B cells in chronic inflammatory bowel disease Gastroenterology *67* 569–577 1974
19. *Tong M J., Wallace A M Peters R. L & Reynolds T B.* Lymphocyte stimulation in hepatitis B infection. New Engl J Med. *293* 318–322 1975
20. *Vittal, S B V., Dourdourekas D., Shobassy N Ainis H Clowdus B.F & Steigmann F* Immunoglobulin and autoantibody response in acute and chronic liver disease. Am. J Med. *57* 546–550 1974
21. *Weeke B.* Carbamylated human immunoglobulins tested by electrophoresis in agarose and antibody containing agarose. Scand. J lab Invest. *21* 351–354 1968.

Acta path. microbiol. scand. Sect. C 85 35–40, 1977

IMMUNE RESPONSE IN MICE TO HAPTEN CONJUGATED SEPHAROSE

SAIJA KOSKIMIES

Department of Serology and Bacteriology, University of Helsinki, Haartmaninkatu 3, 00290 Helsinki 29, Finland

Koskimies, S. Immune response in mice to hapten conjugated Sepharose. Acta path. microbiol. scand. Sect. C, 85: 35–40, 1977.

Mice were injected intraperitoneally with Sepharose 4B beads coupled with hapten NIP and their anti-NIP response was studied by counting antibody forming cells and determining serum titers. Mice responded well to doses of 0.7 ml of packed beads but 0.3 and 1.2 doses induced much weaker responses. Anti-NIP titers in recipients of 0.7 ml of the antigen lasted nearly constant for at least 7 weeks. Both T cell status of the recipient and use of adjuvant had an effect on the response. Antigen without adjuvant induced primarily IgM antibodies in normal mice, but IgM and IgG in nude mice. When *Hemophilus pertussis* or polyacrylic acid was used as adjuvant both normal and nude mice produced IgM and IgG antibodies, and normal mice produced in addition IgA antibodies.

Key words: Immune response; hapten conjugated Sepharose; mice.

S. Koskimies, Department of Serology and Bacteriology, University of Helsinki, Haartmaninkatu 3, 00290 Helsinki 29, Finland.

Received 26. iii.76 Accepted 26.viii.76

Immunogenicity of a hapten directly coupled onto an inert carrier has been studied in cultures of lymphoid cells (13, 17). *Katz & Unanue* (17) found that DNP coupled onto the culture dish did not induce an anti-DNP response but *Feldmann et al.* (13) could get a good response in spleen cell cultures with TNP-polyacrylamide beads. In this study haptenated non-digestable beads have used for immunization of whole animals, mice. Such studies are interesting because of the old postulate that the antigen has to be digestable by hydrolytic enzymes (4). Another reason making such beads interesting immunogens is the fact that they adsorb considerable amounts of antibody. Also, because of their physical uniqueness they may induce different immunoglobulins than conventional antigens. Finally, the effect of T cell deprivation on such immunization was considered worth of a study.

MATERIALS AND METHODS

Antigens. The hapten used in all experiments was 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) coupled to various carrier molecules.

Hapten molecules were conjugated to agarose (Sepharose) using the method of *Cuatrecasas* (6). Sepharose 4B (Pharmacia, Uppsala) was activated with cyanogen bromide, 50 mg per ml of Sepharose. After washing on a sintered glass filter with cold 0.1 M sodiumbicarbonate NIP-ethylenediamine was added in ratio 1.3 mg per 1 ml of packed Sepharose and the mixture was kept at 4° C under constant stirring for 16 h. The NIP-Sepharose conjugate (NIP-Seph) was washed several times with borate buffer pH 8.3, 8M urea, citrate buffer pH 2.6 and carbonate buffer pH 10 in this order. Residual activity was inactivated with 0.05M

ethanolamine for 6 h and the conjugate washed (20 × volume) with borate buffer The relative amount of NIP on Sepharose was measured by spectrophotometer at 430 nm after dissolving a known amount of packed conjugate to 5N HCl. A conjugate containing 1.3×10^{-4}M of NIP per ml of packed Sepharose was used in experiments. A preparation of NIP coupled to ovalbumin containing 7 8 groups of NIP per mole of ovalbumin (NIP-OA) was prepared according to *Brownstone et al* (3) NIP Fab conjugate was a gift from Dr *Marc Feldmann*

Animals and immunization Mice of both sexes of C57BL/6 C57BL/6 *nu/nu* Balb/c *nu/nu strains* were used. They were bred in this laboratory Nude mice and their littermate controls were immunized at the age of one month, others at the age of 3–6 months. All animals were immunized intraperitoneally with either varying doses of NIP-Seph conjugate or 0 1 mg of alum precipitated NIP-OA. Polyacrylic acid 0 1 mg (PAAC) and *Hemophilus pertussis* bacteria 10^9 (H p.) were used as adjuvants in some experiments and were always given i p

Anti NIP-antibody assay The preparation of NIP-conjugated bacteriophage T2 and the measurement of haptenated phage inactivation (HPI) have been described earlier (23) The proportions of IgM and IgG were determined by the combined reduction hapten inhibition method (20) where IgM antibody activity is abolished by 2-mercaptoethanol treatment. The anti-NIP titres given in this report are the mean $\log_{10}$ of the reciprocals of the antibody dilutions necessary to inhibit 50 per cent of the plaque forming units. Sucrose gradient was used to separate physically IgM IgA and IgG components of anti-NIP antibody and is described previously (21) 20 fractions of 16 drops were taken and they were analysed by HPI method Inactivation of IgA and IgG antibodies was performed as follows One volume of rabbit anti-mouse IgA or IgG was mixed with 3 volumes of the serum to be absorbed diluted 1 20 This mixture was kept at +37 C for on hour and at +4 C overnight before it was run in the sucrose gradient.

Hemolytic plaque assay NIP Fab sheep erythrocytes (NIP-SRBC) were prepared for the plaque assay by incubating NIP Fab with 3 times washed SRBCs in ratio 100 µg of NIP Fab per 1 ml of 10 per cent SRBC suspension for one hour at 37 C. Cells were washed three times with saline

TABLE 1 *Anti NIP PFC Response (PFC/10^6 Spleen Cells) in C57Bl/6 and C57Bl/6 Nude Mice Immunized with NIP-Sepharose Conjugate*

Antigen	No of mice	IgM	IgA	IgG
NIP-Seph	11	240	4	5§
NIP-Seph + H.p *	12	798	178	122§
NIP-OA	6	442	18	4028†
NIP-OA + H p	6	738	19	4040†
H p	5	44	3 3	
Uncoupled Seph	5	38	2 1	
Unimmunized	5	25		
NIP-Seph	6 nude	323	4†	
NIP Seph	5 nude	290	1 4	72þ
NIP-Seph + H p.	6 nude	227	3†	
NIP-Seph + H p	5 nude	318	2	54þ

Spleen of mice immunized with 0 7 ml of NIP-Seph were tested on day 4 and those of mice immunized with 0 1 mg of NIP-OA were tested on day 7 Each value is the log mean of the number of mice per group Coefficient of variation was 1 02–1.25 except for values ≦5 it was 1 51–2 57 PFC 10^6 spleen cells against SRBC were 16 ± 1 25 IgA and IgG PFC were assayed with rabbit anti-mouse IgA and IgG antibodies.

* H p = *Hemophilus pertussis*

§ Results are a pool of two experiments a and b a) IgA and IgG PFC were achieved by subtracting the direct PFC from the number of enhanced plaques. b) direct PFC were inhibited with chicken anti-mouse IgM antibody *It caused inhibition of all plaques in the absence of rabbit anti-mouse immunoglobulins.* Both methods yielded similar results.

† Direct PFC were subtracted from the number of enhanced plaques.

þ Direct PFC were inhibited with chicken anti-mouse IgM antibody

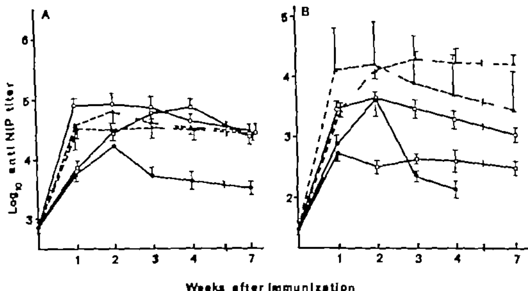


Fig 1 Anti-NIP-titers in mice immunized intraperitoneally with NIP Seph alone or together with an adjuvant. Following doses were used 0.3 ml ●——● 0.7 ml ○——○, 1.2 ml □——□, 0 7 ml + polyacrylic acid 0.1 mg △——△ 0 7 ml + *Hemophilus pertussis cells* 10⁹ ▲- - -▲ Each point represents the log mean of 7-8 mice and its standard error The left hand figure (A) shows the total titer and the right hand figure (B) show the 2 ME resistant titer of the same sera.

and stored in 10 per cent suspension t +4 C. The same cells were used for several assays within one week.

The plaque technique was modification of that developed by *Cunningham & Szenberg* (7) Briefly 0.3 ml of 10 per cent NIP SRBC suspension and 0.1 ml of undiluted guinea pig serum were added to 0.6 ml of RPMI 1640 medium. A 0 4 ml aliquot of this mixture was then mixed with 0.2 ml of the appropriate spleen cell suspension and aliquots of this suspension then pipetted with a Pasteur pipette into slide chambers calibrated to have a capacity of 0.1 ml Chambers were then sealed with paraffin and incubated 50 min at 37 C Plaques were counted under dissection microscope For each assay three chambers were used IgA and IgG indirect plaques were developed by replacing the RPMI 1640 medium with rabbit anti-mouse IgA or IgG with or without chicken anti-mouse IgM. Rabbit anti-mouse immunoglobulins and chicken anti-mouse IgM were prepared in this laboratory as described elsewhere (15 16, 25) Guinea pig complement and anti-immunoglobulins were absorbed t 0 C by incubating the undiluted serum with packed NIP-SRBC in ratio 10 1 The amount of direct plaques is subtracted from the amount of enhanced plaques to ob IgA or IgG plaques in assays where no [illegible] serum was used.

RESULTS

Hemolytic Plaque Forming cell Response to a NIP-Sepharose Conjugate

Mice were immunized with 0 7 ml of NIP Seph with or without *Hemophilus pertussis* bacteria and their spleens were tested on day 4 Another group of mice were similarly immunized using 0 1 mg NIP-OA as antigen and the spleens were tested on day 7 The peak of the responses appeared to be on these days. Normal mice immunized with NIP Seph and adjuvant gave a good response consisting of IgM IgA and IgG classes (Table 1) When immunized without the adjuvant only IgM antibodies could be detected. When the mice were immunized with NIP-OA conjugate the response consisted mainly of IgG PFC, smaller amount of IgM PFC and only few IgA PFC. The use of adjuvant did not affect the magnitude or the class distribution of the response.

Nude mice immunized with NIP Seph generated an IgM response as normal mice.

ethanolamine for 6 h and the conjugate washed (20 × volume) with borate buffer The relative amount of NIP on Sepharose was measured by spectrophotometer at 430 nm after dissolving a known amount of packed conjugate to 5N HCl. A conjugate containing 1 3 × 10 ³M of NIP per ml of packed Sepharose was used in experiments. A preparation of NIP coupled to ovalbumin containing 7 8 groups of NIP per mole of ovalbumin (NIP-OA) was prepared according to *Brownstone et al.* (3) NIP Fab conjugate was a gift from Dr *Marc Feldmann*

Animals and immunization Mice of both sexes of C57BL/6 C57BL/6 nu/nu, Balb/c nu/nu strains were used They were bred in this laboratory Nude mice and their littermate controls were immunized at the age of one month, others at the age of 3–6 months. All animals were immunized intraperitoneally with either varying doses of NIP-Seph conjugate or 0 1 mg of alum precipitated NIP-OA. Polyacrylic acid 0 1 mg (PAAC) and *Hemophilus pertussis* bacteria 10⁹ (H p.) were used as adjuvants in some experiments and were always given i.p

Anti-NIP-antibody assay The preparation of NIP-conjugated bacteriophage T? and the measurement of haptenated phage inactivation (HPI) have been described earlier (23) The proportions of IgM and IgG were determined by the combined reduction hapten inhibition method (20) where IgM antibody activity is abolished by 2-mercaptoethanol treatment. The anti NIP titres given in this report are the mean log_{10} of the reciprocals of the antibody dilutions necessary to inhibit 50 per cent of the plaque forming units. Sucrose gradient was used to separate physically IgM IgA and IgG components of anti NIP antibody and is described previously (21) 20 fractions of 16 drops were taken and they were analysed by HPI method. Inactivation of IgA and IgG antibodies was performed as follows One volume of rabbit-anti-mouse IgA or IgG was mixed with 3 volumes of the serum to be absorbed diluted 1 20 This mixture was kept at +37 C for on hour and at +4 C overnight before it was run in the sucrose gradient.

Hemolytic plaque assay NIP Fab sheep erythrocytes (NIP-SRBC) were prepared for the plaque assay by incubating NIP Fab with 3 times washed SRBCs in ratio 100 μg of NIP Fab per 1 ml of 10 per cent SRBC suspension for one hour at 37 C. Cells were washed three times with saline

TABLE 1 *Anti-NIP PFC Response (PFC/10⁶ Spleen Cells) in C57Bl/6 and C57Bl/6 Nude Mice Immunized with NIP-Sepharose Conjugate*

Antigen	No of mice	IgM	IgA	IgG
NIP-Seph	11	240	4	5§
NIP-Seph + H p *	12	298	178	122§
NIP OA	6	442	18	4028†
NIP-OA + H p	6	738	19	4040†
H p	5	44	3 3	
Uncoupled Seph	5	38	2 1	
Unimmunized	5	25		
NIP-Seph	6 nude	323	4†	
NIP-Seph	5 nude	290	1 4	72þ
NIP-Seph + H p	6 nude	?27	3†	
NIP-Seph + H p	5 nude	318	2	54þ

Spleen of mice immunized with 0 7 ml of NIP-Seph were tested on day 4 and those of mice immunized with 0 1 mg of NIP-OA were tested on day 7 Each value is the log mean of the number of mice per group Coefficient of variation was 1 02–1 ?5 except for values ≦5 it was 1 51 2 57 PFC/10⁶ spleen cells against SRBC were 16 ± 1 25 IgA and IgG PFC were assayed with rabbit anti-mouse IgA and IgG antibodies.

* H p = *Hemophilus pertussis.*

§ Results are a pool of two experiments a and b a) IgA and IgG PFC were achieved by subtracting the direct PFC from the number of enhanced plaques. b) direct PFC were inhibited with chicken anti mouse IgM antibody It caused inhibition of all plaques in the absence of rabbit anti mouse immunoglobulins. Both methods yielded similar results.

† Direct PFC were subtracted from the number of enhanced plaques

þ Direct PFC were inhibited with chicken anti-mouse IgM antibody

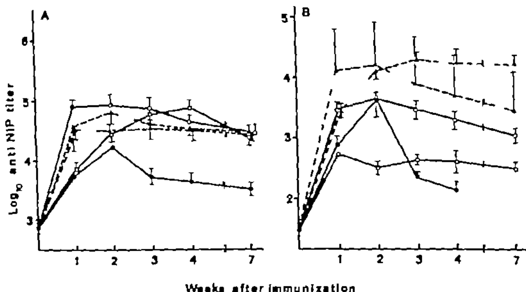


Fig. 1. Anti-NIP-titers in mice immunized intraperitoneally with NIP-Seph alone or together with an adjuvant. Following doses were used: 0.3 ml ●—●, 0.7 ml ○—○, 1.2 ml □—□, 0.7 ml + polyacrylic acid 0.1 mg △—△, 0.7 ml + *Hemophilus pertussis cells* 10⁹ ▲---▲. Each point represents the log mean of 7–8 mice and its standard error. The left hand figure (A) shows the total titer and the right hand figure (B) shows the 2-ME resistant titer of the same sera.

and stored in 10 per cent suspension at +4 C. The same cells were used for several assays within one week.

The plaque technique was a modification of that developed by *Cunningham & Szenberg* (7). Briefly 0.3 ml of 10 per cent NIP-SRBC suspension and 0.1 ml of undiluted guinea pig serum were added to 0.6 ml of RPMI 1640 medium. A 0.4 ml aliquot of this mixture was then mixed with 0.2 ml of the appropriate spleen cell suspension and aliquots of this suspension then pipetted with a Pasteur pipette into slide chambers calibrated to have capacity of 0.1 ml. Chambers were then sealed with paraffin and incubated 30 min at 37 C. Plaques were counted under a dissection microscope. For each assay three chambers were used. IgA and IgG indirect plaques were developed by replacing the RPMI 1640 medium with rabbit anti-mouse IgA or IgG with or without chicken anti-mouse IgM. Rabbit anti-mouse immunoglobulins and chicken anti-mouse IgM were prepared in this laboratory as described elsewhere (15, 16, 25). Guinea pig complement and anti-immunoglobulins were absorbed at 0 C by incubating the undiluted serum with packed NIP-SRBC in ratio 10:1. The amount of direct plaques is subtracted from the amount of enhanced plaques to give IgA or IgG plaques in assay where no anti-IgM serum was used.

RESULTS

Hemolytic Plaque Forming cell Response to a NIP-Sepharose Conjugate

Mice were immunized with 0.7 ml of NIP-Seph with or without *Hemophilus pertussis* bacteria and their spleens were tested on day 4. Another group of mice were similarly immunized using 0.1 mg NIP-OA as antigen and the spleens were tested on day 7. The peak of the responses appeared to be on these days. Normal mice immunized with NIP-Seph and adjuvant gave a good response consisting of IgM, IgA and IgG classes (Table 1). When immunized without the adjuvant only IgM antibodies could be detected. When the mice were immunized with NIP-OA conjugate the response consisted mainly of IgG PFC, smaller amount of IgM PFC and only few IgA PFC. The use of adjuvant did not affect the magnitude or the class distribution of the response.

Nude mice immunized with NIP-Seph generated an IgM response as normal mice,

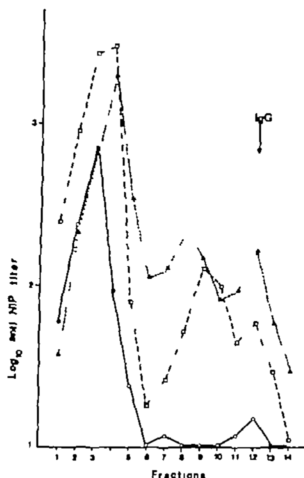


Fig 2 Sucrose gradient fractionation of 14 day anti-NIP sera. Sera from mice immunized with 0.7 ml of NIP-Seph ○—○ NIP-Seph + *Hemophilus pertussis* △ △ or NIP-Seph + polyacrylic acid □- - -□, were run on sucrose gradient and the fractions were tested for anti-NIP antibody activity by HPI method. Anti DNP serum was used as a marker for IgG antibody

Their IgA response was poor with or without adjuvant. The number of IgG PFC was approximately half of that in adjuvant treated normal mice. No adjuvant effect could be demonstrated in nude mice.

Anti NIP Serum Titers in Mice Immunized with NIP-Sepharose

A total of ninety mice received a single but variable dose of antigen with or without an adjuvant. They were bled at intervals for up to seven weeks and the serum antibody titers were determined in the presence or absence 2-mercaptoethanol (2 ME) Serum titers in recipients of an 0 7 ml dose confirmed the findings of Table 1 Adjuvants mainly increased the 2 ME resistant titer The peak titer was usually reached by day 14 and by day 49 it had only dropped to approximately one half of the peak (Fig 1A) This response was rather sensitive to both reduction and increase of the antigen dose. Reduction of the dose by one half reduced the total titer ten fold and made it shorter lasting Also doubling of the optimal dose decreased or delayed the titers.

Immunoglobulin Class Distribution in the Response to NIP-Sepharose

All sera which were tested for total titer of anti NIP antibodies were tested also for

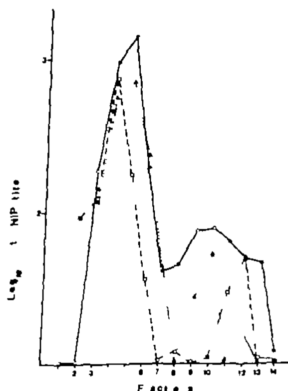


Fig 3 Sucrose gradient analysis of different Ig classes of a 14 day anti-NIP serum. A serum from a mouse immunized with 0 7 ml NIP-Seph and polyacrylic acid was mixed with rabbit anti-mouse IgG or IgA antibody and was run thereafter on a sucrose gradient. Unabsorbed serum ○- -○, serum absorbed with anti IgG antibody △ △ serum absorbed with anti IgA antibody □ - -□. 19S = fractions 4–5 7S = fraction 12.

TABLE 2. *Anti-NIP Antibodies in Nude and Control Mice in Response to NIP-Sepharose and NIP-OA (-on) gates*

Antigen		Nude mice 7*	Nude mice 14	Control mice 7	Control mice 14
NIP-Seph	total	4.35 ± 10	4 17 ± 12	4.07 ± 11	4 63 ± .09
	ME-res	2.62 ± .38	2.64 ± 45	1 43 ± .08	1 71 ± .15
NIP-Seph + PAAC	total	4.34 ± .06	3.96 ± .18	4 03 ± .06	4 64 ± .21
	ME-res	2.33 ± 19	2.12 ± 16	2 15 ± .08	3 16 ± 11
NIP-OA	total	2.81 ± 15	2.83 ± 11	4.37 ± 19	4.99 ± 14
	ME-res	<1 40	1 47 ± .04	3.53 ± 18	4.85 ± 17
Unimmunized	total	2.88 ± .07			
	ME-res	1 49 ± 10			

Balb/c nude mice and their normal littermates were immunized intraperitoneally with 0.7 ml of NIP Seph or 0.1 mg of NIP-OA Each value is the log mean of 5–8 mice and its standard error Both total and 2-mercaptoethanol resistant titers assayed by HPI method are shown.

* Days after immunization.

9 ME resistant antibodies (Fig 1B) In mice immunized with NIP-Seph the relative amount of 2 ME resistant antibody decreased at the peak of the response when the dose of antigen was increased. With doses of 0.5 and 0.7 ml of NIP-Seph the kinetics of the 2 ME resistant antibody followed that of the total titer whereas with 1.2 ml dose the 2 ME resistant titer remained nearly constant through the observation period.

Sucrose gradient analyses were performed to separate different immunoglobulin classes. Four sera from mice immunized with 0 7 ml of NIP Seph, 2 sera from mice similarly immunized together with H.p. and 6 sera from mice immunized together with PAAC were analysed. Anti-DNP serum was used as a marker for IgG peak Results of representative sera are shown in Fig 2. Sera from mice immunized with NIP-Seph contained a major IgM peak (fractions 3–4) and a small IgG peak (fraction 12) Sera from mice immunized with an adjuvant contained a major IgM peak, IgG peak and one additional peak between IgM and IgG (fractions 8–10) When such a serum was incubated with anti-IgG before centrifugation the peak at fraction 12 disappeared. If it was incubated with anti-IgA the intermediate peak disappeared (Fig 3)

Anti NIP Serum Titers in Nude Mice in the Response to NIP-Sepharose

Balb/c nude mice immunized with 0.7 ml of NIP-Seph showed the peak of the response earlier than the control mice. They also showed higher 2 ME resistant titer than the control group (Table 2) When an adjuvant was used in immunization the titers did not change in the nude mice but in the control mice the 2 ME resistant titer increased. The response to NIP-OA conjugate in the nude mice was at the background level but the normal mice gave a good response of both IgM and IgG classes. The serum titers thus confirmed the data obtained in the study of hemolytic plaque forming cells.

DISCUSSION

Results in this report showed that NIP Sepharose, a non-digestible hapten conjugate induced anti-NIP antibodies. The response to one injection of the antigen was long lasting with a rather constant titer This was perhaps caused by the long persistance of the antigen 15 weeks after immunization antigen could still be recovered from the peritoneal cavity The antibody response to pneumo-

coccal polysaccharide SIII is also long lasting (30) probably because the antigen persists fixed in the tissues and is poorly metabolizable (11)

Only a narrow dose range of NIP-Seph was strongly immunogenic. The optimal dose was 0 7 ml and half of this dose caused only one tenth of the titers that 0 7 ml induced. A supraoptimal dose of 1 2 ml induced delayed and somewhat lower response than the dose of 0 7 ml

Particularly the 2 ME resistant antibody response was sensitive to changes of the dose By increasing the dose the relative amount of 2 ME resistant antibody was decreased. With supraoptimal dose the titer remained at a constant low level. These results are in agreement with the results obtained with TNP keyhole limpet hemocyanin conjugated Sepharose (TNP KLH-Seph) in vitro as well as with SIII and DNP lys-SIII in vivo which all show a narrow dose range in immunogenicity (1 13 18) Protein antigens and SRBC have a wider range of optimal doses (10 11 26)

The adsorption capacity of the NIP-Seph conjugate could explain the delay of the peak response and the low level of IgG antibodies. The antigen probably adsorbs the early antibodies and in particular the IgG antibodies. An injection of 1.2 ml to a mouse that was undergoing a secondary anti NIP response (mainly IgG) caused a 90 per cent drop in anti NIP titer (M Kaartinen pers. communication)

In preliminary experiments not reported here several NIP-Seph conjugates with different hapten densities were tested The antigen showed a narrow peak on epitope desity curve too When the amount of hapten per bead was decreased to half of the optimal density the titer dropped to one tenth When the hapten density was decreased to one tenth of the optimal the conjugate induced hardly any response. Similarly the increase of the hapten concentration per bead resulted to a decrease of the titer This is in accordance with the results found with TNP KLH-Seph (13) These considerations may provide an explanation for the failure of others to demonstrate immunogenicity of insolubilized antigen (17)

NIP-Seph has characteristics of a T independent antigen. Its physical form shares the properties of other T independent antigens it is polymeric with high epitope density and it is poorly metabolizable. It induced equal amounts of IgM antibodies both in normal and in nude mice but a slightly lower level of IgG antibodies in normal than in nude mice. These results were obtained both at the PFC level and at the serum antibody level. Earlier investigators have observed that small amounts of IgG antibodies can be produced without mature T cells to some other T independent antigens like TNP KLH-Seph, TNP Ficoll SIII and DNP AE-Dextran (2, 13 28 29) Immunization of anti-thymocyte serum treated or nude mice with SIII or DNP AE-Dextran generated even higher IgG responses than immunization of control mice (2 28) These findings correspond to the higher IgG response of nude mice than the control mice to NIP-Seph

The response induced by NIP-Seph with adjuvants, *Hemophilus pertussis* or poly acrylic acid had characteristics of a T cell dependent response. Adjuvants increased the IgG response and augmented the IgA response to NIP-Seph conjugate in normal mice Adjuvants had no effect on the IgM response in normal mice or any class of response in nude mice These results serve as an additional confirmation of the relatively greater dependence of IgA and IgG antibody synthesis than IgM antibody synthesis on T cells

Experimental data by several authors propose that the adjuvant effect of *Hemophilus pertussis* either requires T cells or that *Hemophilus pertussis* has an effect to T cells in addition to B cells (12 14 22 24 27 31) Maillard & Bloom suggest that the adjuvant effect is mediated by an immunologically specific T cell response to antigens on the adjuvant (22) Dresser demonstrated that the effect of T cells and adjuvant together was more than additive suggesting that *Hemo-*

philus pertussis acted on T cells. He also showed that T cells and adjuvant together had greater effect on IgG antibody than on IgM antibody production (12). This agrees with my results.

Polyacrylic acid enhance both IgM and IgG responses to sheep red blood cells in normal mice (8) and it can restore the immune response to this antigen in thymectomized irradiated and bone marrow protected mice (9). In experiments reported here it enhanced the response to NIP-Seph only in the presence of T cells and thus behaved similarly to *Hemophilus pertussis*.

The association of a B cell and a haptenated bead was sufficient to induce hapten specific IgM response and to some extent IgG antibody production too. IgA and most of the IgG precursor cells became stimulated with NIP-Seph only in the presence of a T cell signal which was induced by an adjuvant. This second signal probably was nonspecific. This is supported by the finding that T independent DNP-lys-SIII, DNP lys-levan and DNP-AE-Dextran display the ability to elicit IgG response in the presence of a T cell signal mediated by graft versus-host reaction (19, 28) and that ConA induced nonspecific T cell product enhances the immune response of Dextran primed spleen cells in vitro (5).

This study was supported by the *Medical Research Council, Academy of Finland*.

REFERENCES

1. *Baker, P. J., Stashak, P. W., Amsbaugh, D. F. & Prescott, B.*: Characterization of the antibody response to type III pneumococcal polysaccharide at the cellular level. I. Dose response studies and the effect of prior immunization on the magnitude of the antibody response. Immunology *20*, 469–480, 1971.
2. *Barthold, D. R., Prescott, B., Stashak, P. W., Amsbaugh, D. F. & Baker, P. J.*: Regulation of the antibody response to type III pneumococcal polysaccharide. J. Immunol. *112*, 1042–1050, 1974.
3. *Brownstone, A., Mitchison, N. A. & Pitt-Rivers, R.*: Chemical and serological studies with an iodine containing synthetic immunological determinant 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) and related compounds. Immunology *10*, 465–479, 1966.
4. *Campbell, D. H. & Bulman, N.*: Some current concepts of the chemical nature of antigens and antibodies. Fortchr. Chem. Org. Naturst. *9*, 443–447, 1952.
5. *Chen, J. C. & Lara, M. A.*: The immune response to dextran in Balb/c mice. I. Modification of "thymus-independent" response by the T cell mitogen concanavalin A. J. Immunol. *116*, 416–422, 1976.
6. *Cuatrecasas, P.*: Protein purification by affinity chromatography. Derivatizations of agarose and polyacrylamide beads. J. Biol. Chem. *245*, 3059–3065, 1970.
7. *Cunningham, A. J. & Szenberg, A.*: Further improvements in the plaque technique for detecting single antibody-forming cells. Immunology *14*, 599–609, 1968.
8. *Diamantstein, T., Wagner, B., Beyse, I., Odenwald, M. W. & Schulz, G. S.*: Stimulation of humoral antibody formation by polyanions. I. The effect of polyacrylic acid on the primary immune response in mice immunized with sheep red blood cells. Eur. J. Immunol. *1*, 335–340, 1971.
9. *Diamantstein, T., Wagner, B., L'Age-Stehr, J., Beyse, I., Odenwald, M. V. & Schulz, G. S.*: Stimulation of humoral antibody formation by polyanions. III. Restoration of the immune response to sheep red blood cells by polyanions in thymectomized and lethally irradiated mice protected with bone marrow cells. Eur. J. Immunol. *1*, 302–304, 1971.
10. *Dixon, F. J., Jacot-Guillarmod, H. & McConahey, P. J.*: The antibody response of rabbits and rats to hemocyanin. J. Immunol. *97*, 350–355, 1966.
11. *Dixon, F. J., Maurer, P. H. & Weigle, W. O.*: Immunologic activity of pneumococcal polysaccharide fixed in the tissues of the mouse. J. Immunol. *74*, 188–191, 1955.
12. *Dresser, D. W.*: The role of T cells and adjuvant in the immune response of mice to foreign erythrocytes. Eur. J. Immunol. *2*, 50–57, 1972.
13. *Feldmann, M., Greaves, M. F., Parker, D. C. & Rittenberg, M. B.*: Direct triggering of B lymphocytes by insolubilized antigen. Eur. J. Immunol. *4*, 591–597, 1974.
14. *Hamaoka, T. & Katz, D. H.*: Cellular site of action of various adjuvants in antibody responses to hapten-carrier conjugates. J. Immunology *111*, 1554–1563, 1973.
15. *Imanishi, T., Hurme, M., Sarvas, H. & Mäkelä, O.*: Expression of a mouse Ig V gene

in antibodies of three immunoglobulin classes. Eur Immunol. 5 198–202 1975

16. *Imanishi T & Mäkelä O* Strain differences in the finespecificity of mouse antihapten antibodies. Eur J Immunol. 3 323–330 1973
17. *Katz D H & Unanue E. R* Critical role of determinant presentation in the induction of specific responses in immunocompetent lymphocytes. J exp Med. 37 967–990 1973
18. *Klaus G G B. & Humphrey J H* The immunological properties of haptens coupled to thymus-independent carrier molecules. I Characteristics of the immune responses to dinitro-phenyllysine-substituted pneumococcal polysaccharide (SIII) and levan. Eur J Immunol. 4 370–377 1974
19. *Klaus G G B & McMichael A J* The immunological properties of hapten coupled to thymus-independent carrier molecules. II The influence of the graft versus-host reaction on primary antibody responses to hapten-coupled polysacchandes and proteins. Eur J Immunol 4 505–511 1974
20. *Kontiainen S & Mäkelä, O* Determination of 19S and 7S components in an anti-hapten antibody Ann. Med. Exp Fenn. 45 472–476 1967
21. *Koskimies S Sarvas H & Kaartinen M* Ig class distribution in anti hapten responses to hapten-erythrocyte conjugates. Immunol Comm. 2 1–14 1973
22. *Maillard J & Bloom B R* Immunological adjuvants and the mechanism of cell coopera tion. J exp. Med. 136 185 1972
23. *Mäkelä O* Assay of anti hapten antibody with the aid of hapten coupled bacteriophage. *Immunology 10 81–86 1966.*
24. *Morse S I & Riester S K* Studies on the leucocytosis and lymphocytosis induced by *Bordetella pertussis* II The effect of pertussis vaccine on the thoracic duct lymph and lymphocytes in mice. J exp. Med. 125 619–629 1967
25. *Nakamura, I Ray A & Mäkelä, O.* Oligomeric IgA The major component of the in vitro primary response of mouse spleen fragments. J exp Med 138 973–988, 1973
26. *Nossal G J V Austin C M & Ada G L.* Antigens in immunity VII. Analysis of immunological memory Immunology 9 333–348, 1965
27. *Phanuphak P Moorhead J F & Clemen, H N* Immunologic activities of pertussis-treated lymphocytes. Int. Arch. Allergy 43 305–316 1972.
28. *Rüde E. Wrede J & Gundelach, M L.* Production of IgG antibodies and enhanced response of nude mice to DNP AE-Dextran. J Immunol. 116 527–533 1976.
29. *Sharon R. McMaster P R. B. Kask A M Owens J D & Paul W E.* DNP Lys-Ficoll A T independent antigen which elicits both IgM and IgG anti DNP-antibody-selecting cells. J Immunol. 114 1585–1589 1975
30. *Sukind G W Paul W E & Benacerraf B.* Use of radioactively labeled SIII to study the blood clearance and the immune response of mice to pneumococcal polysaccharides. Immunochemistry 4 455–465 1967
31. *Taub R N & Gershon R K* The effect of localized injection of adjuvant material on the draining lymph node. J Immunol. 108 377–386 1972.

Acta path. microbiol. scand. Sect. C, 85 41–48, 1977

LYMPHOCYTE SUBPOPULATIONS IN CROHN'S DISEASE AND CHRONIC ULCERATIVE COLITIS

S. Freiesleben Sørensen and Lene Høj

Department of Medicine B (gastro-enterology), Gentofte Hospital, University of Copenhagen and the Department of Medicine TA, Rigshospitalet, University of Copenhagen, Denmark

Sørensen, S. F. & Høj, Lene. Lymphocyte subpopulations in Crohn's disease and chronic ulcerative colitis. Acta path. microbiol. scand. Sect. C, 85 41–48, 1977

Lymphocyte subpopulations in peripheral blood were studied by the sheep red blood cell rosette technique for the identification of T-lymphocytes, and immunofluorescence staining with rabbit anti-human immunoglobulin sera for the identification of B-lymphocytes. In Crohn's disease, the total number of lymphocytes was found to be reduced and an almost equal reduction in all the lymphocyte subpopulations studied was found. In ulcerative colitis neither the number of T-lymphocytes nor of B-lymphocytes were found to differ significantly from normal values and the same was true of all B-lymphocyte subpopulations. However, the number of lymphocytes carrying neither surface immunoglobulin nor sheep red blood cell receptors was found to be significantly increased. The results were correlated to clinical data.

Key words: T-lymphocytes; B-lymphocytes; Crohn's disease; chronic ulcerative colitis.

S. Freiesleben Sørensen, Department of Medicine TA, Rigshospitalet, Tagensvej 18, DK 2200 Copenhagen N, Denmark.

Received 2.ix.76 Accepted 2.ix.76

In recent years much interest has been focused on the immunological mechanisms involved in the pathogenesis of inflammatory bowel disease (*Bendixen* 1969, *Kirsner* & *Goldgraber* 1969), but conclusive findings are lacking. Most studies of cellular immunity in ulcerative colitis have not revealed a depressed immune competence (*Hinz et al.* 1967, *Parent et al.* 1971, *Pinedo* & *Watson* 1971, *Binder et al.* 1966). In contrast, several studies of the cell-mediated immunity in patients with Crohn's disease have shown a reduced immune competence. Reduced cutaneous delayed hypersensitivity and lymphocyte hyporesponsiveness to non-specific mitogens and allogeneic cells have been reported by several authors (*Jones* 1969, *Walker* 1969, *Parent et al.* 1971, *Richens* 1974). It applies to both diseases, however, that the results published are conflicting (*Sachar* 1973, *Aas* 1972, *McHattie* 1971, *Asquith* 1973, *Röpke* 1972).

The purpose of the present study was to determine the total peripheral lymphocyte count and the size of lymphocyte subpopulations in patients with ulcerative colitis and Crohn's disease, and to compare the results with those obtained in healthy controls. Furthermore, it was the purpose to correlate the results with clinical data.

in antibodies of three immunoglobulin classes. Eur Immunol *5* 198–202 1975

16 *Imanishi T & Mäkelä O* Strain differences in the finespecificity of mouse antihapten antibodies. Eur J Immunol *3* 323–330 1973

17 *Katz D H & Unanue E R* Critical role of determinant presentation in the induction of specific responses in immunocompetent lymphocytes. J exp Med. *37* 967–990 1973

18 *Klaus G G B & Humphrey J H* The immunological properties of haptens coupled to thymus independent carrier molecules. I Characteristics of the immune responses to dinitro-phenyllysine-substituted pneumococcal polysaccharide (SIII) and levan. Eur J Immunol. *4* 370–377 1974

19 *Klaus G G B & McMichael A J* The immunological properties of hapten coupled to thymus-independent carrier molecules. II The influence of the graft versus-host reaction on primary antibody responses to hapten-coupled polysaccharides and proteins. Eur J Immunol *4* 505–511 1974

20 *Kontiainen S & Mäkelä O* Determination of 19S and 7S components in an anti-hapten antibody Ann. Med. Exp Fenn. *45* 472–476 1967

21 *Koskimies S Sarvas H & Kaartinen M* Ig class distribution in anti-hapten responses to hapten-erythrocyte conjugates. Immunol. Comm. 2 1–14 1973

22 *Maillard J & Bloom B R* Immunological adjuvants and the mechanism of cell cooperation. J exp. Med. *136* 185 1972

23 *Mäkelä O* Assay of anti hapten antibody with the aid of hapten coupled bacteriophage. Immunology *10* 81–86 1966

24 *Morse S I & Riester S A.* Studies on the leucocytosis and lymphocytosis induced by *Bordetella pertussis.* II The effect of pertussis vaccine on the thoracic chick lymph and lymphocytes in mice. J exp. Med. *125* 619-629 1967

25 *Nakamura I Ray A & Mäkelä O* Oligomeric IgA The major component of the in vitro primary response of mouse spleen fragments. J exp Med. *138* 973–988, 1973

26 *Nossal G J V Austin C M & Ada G.L.* Antigens in immunity VII Analysis of immunological memory Immunology *9* 333–348, 1965

27 *Phanuphak P Moorhead J F & Claman H N* Immunologic activities of pertussis-treated lymphocytes. Int. Arch. Allergy *43* 305–316 1972.

28 *Rüde E Wrede J & Gundelach M L.* Production of IgG antibodies and enhanced response of nude mice to DNP-AE-Dextran. J Immunol *116* 527–533 1976.

29 *Sharon R McMaster P R B. Kask A M Owens J D & Paul W E.* DNP Lys-Ficoll A T independent antigen which elicits both IgM and IgG anti DNP antibody-selecting cells. J Immunol *114* 1585–1589 1975.

30. *Siskind G W Paul W E & Benacerraf B.* Use of radioactively labeled SIII to study the blood clearance and the immune response of mice to pneumococcal polysaccharides. Immunochemistry *4* 455–465 1967

31 *Taub R N & Gershon R K* The effect of localized injection of adjuvant material on the training lymph node. J Immunol. *108* 377–386, 1972.

Acta path. microbiol. scand. Sect. C, 85 41–48, 1977

LYMPHOCYTE SUBPOPULATIONS IN CROHN'S DISEASE AND CHRONIC ULCERATIVE COLITIS

S. Freiesleben Sørensen and Lene Hoj

Department of Medicine B (gastro-enterology) Gentofte Hospital, University of Copenhagen and the Department of Medicine TA, Rigshospitalet, University of Copenhagen, Denmark

Sørensen, S. F & Høj, Lene. Lymphocyte subpopulations in Crohn's disease and chronic ulcerative colitis Acta path. microbiol. scand. Sect. C, 85 41–48, 1977

Lymphocyte subpopulations in peripheral blood were studied by the sheep red blood cell rosette technique for the identification of T-lymphocytes, and immunofluorescence staining with rabbit anti-human immunoglobulin sera for the identification of B-lymphocytes. In Crohn's disease, the total number of lymphocytes was found to be reduced and an almost equal reduction in all the lymphocyte subpopulations studied was found. In ulcerative colitis neither the number of T-lymphocytes nor of B-lymphocytes were found to differ significantly from normal values and the same was true of all B-lymphocyte subpopulations. However the number of lymphocytes carrying neither surface immunoglobulin nor sheep red blood cell receptors was found to be significantly increased. The results were correlated to clinical data.

Key words T-lymphocytes B-lymphocytes Crohn's disease chronic ulcerative colitis.

S. Freiesleben Sørensen, Department of Medicine TA, Rigshospitalet, Tagensvej 18, DK-2200 Copenhagen N Denmark

Received 2.ix.76 Accepted 2.ix.76

In recent years much interest has been focused on the immunological mechanisms involved in the pathogenesis of inflammatory bowel disease (*Bendixen* 1969 *Kirsner & Goldgraber* 1969) but conclusive findings are lacking. Most studies of cellular immunity in ulcerative colitis have not revealed a depressed immune competence (*Hinz et al.* 1967 *Parent et al* 1971 *Pinedo & Watson* 1971 *Binder et al.* 1966) In contrast, several studies of the cell-mediated immunity in patients with Crohn's disease have shown a reduced immune competence Reduced cutaneous delayed hypersensitivity and lymphocyte hyporesponsiveness to non-specific mitogens and allogeneic cells have been reported by several authors (*Jones* 1969 *Walker* 1969 *Parent et al.* 1971 *Richens* 1974) It applies to both diseases, however that the results published are conflicting (*Sachar* 1973 *Aas* 1972 *McHattie* 1971 *Asquith* 1973 *Röpke* 1972)

The purpose of the present study was to determine the total peripheral lymphocyte count and the size of lymphocyte subpopulations in patients with ulcerative colitis and Crohn's disease, and to compare the results with those obtained in healthy controls. Furthermore, it was the purpose to correlate the results with clinical data.

TABLE 1 *Clinical Data Applying to Patients with Crohn's Disease and Ulcerative Colitis*

	Age (years) Median (range)	Sex F	Sex M	Duration of disease (years) Median (range)	Salicylazo sulphapyridine +	Salicylazo sulphapyridine −	Activity of disease Light, moderate	Activity of disease Inactive	Level of diagnosis* A	Level of diagnosis* B	Level of diagnosis* C	N
Crohn's disease	32 (20–66)	10	6	9 (2–25)	14	2	12	4	12	1	3	16
Ulcerative colitis	42 (21–67)	8	8	9 (1–39)	11	5	13	3				16
Controls	29 (21–55)	11	5									16

* A Crohn's disease. B Probably Crohn's disease. C Possibly Crohn's disease.

PATIENTS AND METHODS

Selection of Patients

Sixteen patients with Crohn's disease involving the small and/or large bowel were examined. The diagnosis was established according to criteria previously defined (*Hoj et al.* 1973). In 11 cases the diagnostic delimitations were based on histological criteria, in the remaining cases they were based on clinical, radiological and endoscopic criteria. Ten patients were females, 6 males; the median age was 32 years (range 20–66). Duration of disease ranged from 2 up to 25 years (median 9 years). In 12 patients, the disease was in an active stage at the time of investigation (abdominal pain and/or diarrhoe (>2 bowel movements per day)). Fourteen patients received salicylazosulphapyridine.

The diagnosis of ulcerative colitis could be established if 3 out of the 4 diagnostic criteria were present (history, endoscopic appearance, cytological/histological findings, radiological appearance) as defined by *Anthonisen & Riis* (1971) and *Riis & Anthonisen* (1968). The series included 8 females and 8 males, the median age of whom was 42 years (range 21–67). In thirteen patients, the disease was active at the time of examination and 11 received salicylazosulphapyridine. Duration of disease ranged from less than one year up to 39 years (median 9 years).

No patients received corticosteroids or cytostatics, and patients in whom the disease was in a very active stage were not included. The patients entered the study consecutively from the out-patient's clinic. The control series included 16 healthy volunteers who were working in the hospital and who had not been suffering from any diseases during the last month before investigation. The median age was 29 years (range 21–55). Five were male, 11 were females (Table 1).

Determination of Lymphocyte Subpopulations

The total number of lymphocytes was calculated on the basis of a leucocyte count and a differential count, using routine techniques. Lymphocytes and monocytes were isolated from heparinized blood by differential centrifugation. A mixture of blood and Hanks' solution (1:2) was layered on top of Lymphoprep (Nyegaard and Co A/S, Oslo) and centrifuged (400 G, 30 min). Monocytes and lymphocytes were harvested from the interphase and, after 3 washes, they were incubated with latex particles at 37° C for 30 min in order to label the monocytes. It had been shown in preliminary experiments that 12 per cent (range 5–18) of the mononuclear cells could be identified as monocytes by this method.

T-lymphocyte Quantitation Using the Sheep Red Blood Cell (SRBC) Rosette Technique

After incubation with latex particles, the cells were washed twice in Hanks' solution and the cell concentration was adjusted to 3×10^6/ml. 100 µl of this suspension was mixed with 100 µl of a 0.5 per cent suspension of SRBC which had been washed 3 times in Hanks' solution before use. Human AB-serum, absorbed twice with SRBC's was added to a final concentration of 10 per cent.

After incubation for 30 min at 37° C and for 18 h at 4° C, the cells were resuspended by cautious rotation of the tube between two hands upon which the fraction of rosette forming lymphocytes was determined. At least 200 lymphocytes were counted and lymphocytes to which 3 or more SRBC's were attached were counted as T lymphocytes.

Immunofluorescence Test

Fluorescein-conjugated rabbit anti-human immunoglobulin sera were purchased from Dacopatts A/S, Copenhagen. Six different sera were used

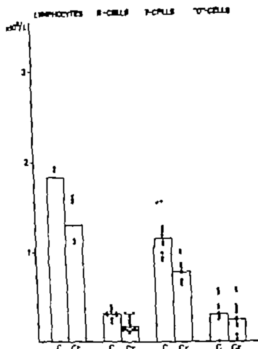


Fig. 1 Lymphocyte subpopulations in Crohn disease. Columns indicate median values. C Controls. Cr Patients with Crohn disease

1 A polyvalent antiserum against IgG IgA, IgM, kappa and lambda.
2 Antiserum against IgG specific for γ-chains.
3 Antiserum against IgA specific for α-chains.
4 Antiserum against IgM specific for μ-chains.
5 Antiserum against kappa light chains.
6 Antiserum against lambda light chains.

After incubation with latex particles, the cells were spun down and 3 drops of antiserum dilution were added to the cell buttons. The tubes were incubated at 0° C for 30 minutes and after 3 washes at 4 C, the cells were examined for the presence of membrane fluorescence. At least 200 lymphocytes in each preparation were counted.

For the sake of convenience, cells stained with the polyvalent antiserum are referred to as B-lymphocytes (see discussion)

The fraction of cells which remained after subtraction from 100 of the percentage of T-lymphocytes and the percentage of B-lymphocytes is referred to as "O"-lymphocytes

The yield of lymphocyte was usually about 80 per cent and always above 60 per cent

Details of the method have been given elsewhere (*Sorensen et al.* 1976)

The statistical test used was Mann-Whitney rank sum test.

RESULTS

In *Crohn's disease* a reduction in the total number of circulating lymphocytes was found ($P<0.05$) Comparison of the median percentage of each lymphocyte subpopulation in patients and in controls showed no significant differences. It could therefore be concluded that all subpopulations were equally involved in the lymphopenia (see Figs. 1 and 2 and Table 2)

Any correlation between duration of disease and the size of any of the lymphocyte subpopulations could not be found. Owing to the low number of patients with inactive disease no meaningful comparison between lymphocyte subpopulation and disease activity could be made.

In cases of *ulcerative colitis* the total number of lymphocytes did not differ significantly from that in controls. The levels of T and B-

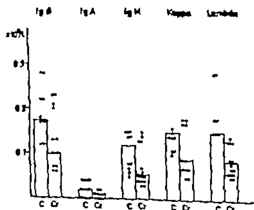


Fig. 2 B-lymphocyte subpopulations in Crohn disease. Columns indicate median values. IgG IgA, IgM kappa and lambda refer to lymphocytes identified with the corresponding antisera. C Controls. Cr Patients with Crohn's disease.

TABLE 2. *Lymphocyte Subpopulations in Crohn's Disease and Chronic Ulcerative Colitis*

	Controls	Crohn's disease	Chronic ulcerative colitis
Total no. of lymphocytes	1.83 (0.74–3.53)	1.30 (0.70–2.65)	2.01 (0.62–3.48)
B-Lymphocytes	17 (13–22)* 0.31 (0.11–0.49)‡	14 (8–22) 0.17 (0.11–0.38)	14 (11–22) 0.26 (0.07–0.50)
IgG	10 (6–14) 0.18 (0.08–0.28)	10 (3–13) 0.10 (0.06–0.30)	8 (6–18) 0.19 (0.04–0.39)
IgA	1 (0–2) 0.02 (0.00–0.17)	1 (0–2) 0.01 (0.00–0.06)	1 (0–2) 0.01 (0.00–0.04)
IgM	6 (3–10) 0.12 (0.02–0.28)	6 (2–10) 0.06 (0.03–0.17)	5 (1–8) 0.08 (0.02–0.26)
Kappa	9 (5–14) 0.15 (0.06–0.27)	8 (2–11) 0.09 (0.03–0.19)	7 (5–12) 0.14 (0.03–0.35)
Lambda	8 (5–12) 0.15 (0.04–0.28)	7 (4–12) 0.09 (0.05–0.22)	7 (4–9) 0.13 (0.04–0.30)
T-lymphocytes	67 (48–85) 1.15 (0.40–2.35)	6[illegible] (33–79) 0.78 (0.23–1.26)	56 (2[illegible]–67) 1.08 (0.29–2.17)
"O"-lymphocytes	17 (11–35) 0.31 (0.00–0.78)	20 (3–48) 0.25 (0.08–0.67)	28 (17–56) 0.64 (0.17–0.90)

* per cent median (range)
‡ total $\times 10^9$/L, median (range)

lymphocytes were also normal, but "O" lymphocytes were twice as numerous in patients as in controls (see Figs. 3 and 4 and Table 2) ($P<0.01$). Any correlation between levels of 'O' -cells and activity or duration of the disease could not be demonstrated. Eleven patients received salicylazosulphapyridine while 5 patients were left untreated. The median number of T lymphocytes was 1.37×10^9/L in patients receiving treatment while the corresponding value was 0.45×10^9/L in patients left untreated. This difference was significant ($P<0.01$).

DISCUSSION

The method employed for the enumeration of B-lymphocytes provides an internal control. It would be expected that the sum of cells with surface kappa and lambda light chains would equal the sum of cells with surface IgG, IgA and IgM which again would be expected to equal the number of cells stained with the polyvalent antiserum. The median values in the groups of controls and patients (Table 2) are in agreement with this prediction.

It has recently been suggested however that immunofluorescent labelling of lymphocytes using whole rabbit anti human Ig antibodies does not necessarily identify B-lymphocytes exclusively if the latter are defined as cells carrying surface immunoglobulin receptors produced by the cell. Incubation and washing of the cell suspensions at 37 C or incubation of the cells at low pH reduces the number of cells identified by anti IgG sera, whereas the fraction of cells stained with anti IgM sera remains constant. (*Kumagai et al* 1975, *Lobo et al* 1975). *Winchester et al* (1975) compared the number of cells identified by fluorescent whole rabbit antibody and the corresponding $F(ab)_2$ fragments and found no difference when anti IgM (or IgD) antibody were used. However the number of cells to be identified by the $F(ab)_2$ fragment of the anti IgG antibody was very low as compared with that to be identified by the whole anti IgG antibody.

Altogether these findings suggest that IgG bound to mononuclear cells may be retained during washing procedures while they are released if the cells are incubated with the fluorescent rabbit antibody. Complexes to be

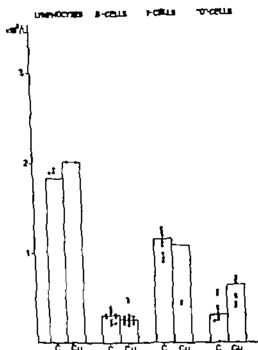


Fig. 3. Lymphocyte subpopulations in chronic ulcerative colitis. Columns indicate medium values. C: Controls. Cu: Patients with ulcerative colitis.

formed between human IgG and rabbit anti human-IgG may subsequently be bound to Fc receptor bearing cells through the Fc parts of the rabbit antibody. This could lead to a false identification of such cells as surface IgG bearing lymphocytes. Furthermore, even if F(ab) fragments of the fluorescent rabbit antibody were used, the method naturally fails to reveal whether the Ig is passively absorbed or it is produced by the cell.

Most likely the results obtained in the present study using the whole anti IgM antibody may represent lymphocytes with true surface IgM. This is probably also true of cells indentified by the whole anti-IgA antibody but the published results on this subject are conflicting (*Kumagai et al.* 1975, Lobo *et al.* 1975). Although the cell suspensions were incubated at 37 C for 30 minutes, it cannot be excluded that the other antisera, in addition to lymphocytes with the corresponding surface specificities, stain cells with Fc receptors. However the above mentioned finding that the sum of cells labelled with the anti-kappa and anti lambda antibodies equals the number of cells labelled with the polyvalent antibody speaks against this possibility.

Malnutrition (*McFarlane & Hamid* 1973), recent operation (*Riddle & Berenbaum* 1967, *Park et al.* 1971, *Andersen et al.* 1976), treatment with steroids (*Yu et al.* 1974a, *Fauci & Dale* 1974) and cytostatica (*Yu et al.* 1974b, *Hurd & Giuliano* 1975) are known to influence the levels of lymphocytes in peripheral blood and their function. Accordingly patients in whom any of these factors were present were excluded from the trial. Moreover patients in whom the disease was in a very active stage were excluded in order to eliminate anemia, weight loss, intraabdominal abscesses and fever on the consideration that these phenomena might influence the

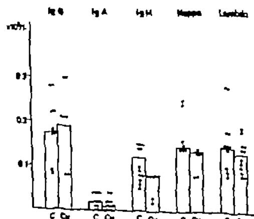


Fig. 4. B-lymphocyte subpopulations in chronic ulcerative colitis. IgG, IgA, IgM, kappa and lambda refer to lymphocytes identified with the corresponding antisera. C: Controls. Cu: Patients with ulcerative colitis.

level of peripheral lymphocytes in a nonspecific way. This may explain why the results obtained by *Strickland et al* (1974) differed from those obtained in the present study.

Strickland et al found that levels of lymphocytes in the peripheral blood were normal in Crohn's disease. Regarding the lymphocyte subpopulations these authors found a decreased number of T lymphocytes whereas the number of cells with surface IgM and IgA was increased. Since we found an almost equal decrease in all lymphocyte subpopulations our results are in agreement with these obtained by *Bird & Britton* (1974) who found that the *percentage* of T and B-lymphocytes in patients and controls did not differ. The reason why lymphopenia occurs in Crohn's disease is unknown. In view of the independent pathways of the maturation of B and T lymphocytes, the uniform reduction in both lymphocyte subpopulations could point towards a nonselective loss of lymphocytes in this disease. An enteric loss of lymphocytes in the gut affected in the course of Crohn's disease has been shown beyond doubt (*Douglas et al* 1975) and at present this hypothesis seems to be the most attractive explanation of the low lymphocyte level in Crohn's disease.

Strickland et al (1975) have reported that the number of T and B-lymphocytes in the diseased bowel wall is increased in Crohn's disease. Therefore, a sequestration of lymphocytes in the gut might lead to a drainage of these cells from the peripheral blood and be another possible explanation of our findings.

Our findings in cases of ulcerative colitis are in agreement with the results obtained by *Strickland et al* (1974) except that the latter authors found increased levels of cells with surface IgM and IgA. Furthermore these authors suggested that the level of T lymphocytes might be reduced in ulcerative colitis. This could not be confirmed in the present study but the demonstration of the fact that T lymphocyte counts were distinctly higher in patients treated with salicylazosulphapyridine who represented the majority in the present study indicates that levels of T lymphocyte actually are decreased in patients with untreated ulcerative colitis. However treatment with salicylazosulphapyridine *per se* seems not to influence the levels of T or B-lymphocytes in blood (*Thayer et al* 1976).

Lymphocytes from patients with inflammatory bowel disease have been shown specifically to lyse colonic epithelial cells *in vitro*. The cells responsible for this effect seem to be neither SRBC receptor nor surface Ig-bearing cells. It has been suggested, however that the responsible cells carry Fc receptors, which have been armed *in vivo* by complexes of colonic antigen and anti-colonic antibody (for references see *Stobo et al* 1976).

In the present study the number of "O" lymphocytes was found to be significantly increased in patients with ulcerative colitis. Owing to the technical factors mentioned above it is at present difficult to interprete this finding. The "O" lymphocytes might be Fc receptor bearing cells which could not be identified because SRBC-receptors or sufficient amount of surface Ig were lacking. If antisera with anti IgG specificity normally stain Fc receptor bearing cells, these cells might escape detection in the patients because the Fc receptors are blocked by immunecomplexes. Further studies with a view to solving these questions are in progress.

This study was supported by the *Danish State Medical Research Council*, Grant no. 512 3110 and 512-4359. The technical assistance of *Anne Ambjørnsen* and *Anne Mollmann* is gratefully acknowledged.

REFERENCES

Andersen V, Cohn J & Sorensen S F. Immunological studies in children before and after splenectomy. Acta pædiatr Scand 65, 409–415, 1976.

Aukonsen P & Riis P. Eosinophilic granulocytes in the rectal mucus of patients with ulcerative colitis and Crohn's disease of the ileum and colon. Scand. J Gastroent. 6, 731–734, 1971.

Asquith P Lymphocyte responses to nonspecific mitogens in inflammatory bowel disease. Gastroenterology *65* 1–7 1973

Bendixen G Cellular hypersensitivity to components of intestinal mucosa in ulcerative colitis and Crohn disease. Gut. *10* 631–636, 1969

Bender H J Spero H M & Thayer W R Jr Delayed hypersensitivity in regional enteritis and ulcerative colitis. Am. J Dig. Dis. *11* 572–574 1966.

Bird A. G & Britton S No evidence for decreased lymphocyte reactivity in Crohn disease. Gastroenterology *67* 926–932, 1974.

Douglas A. P Weetman, A P & Haggith J Enteric loss of lymphocytes in celiac disease and in Crohn's disease. Gastroenterology *68* 1056 (Abstr) 1975.

Fauci, A S & Dale D C The effect of in vivo hydrocortisone on subpopulations of human lymphocytes. J Clin Invest. *53* 240–246, 1974

Hinz, C F J Perlmann P & Hammarström S Reactivity in vitro of lymphocytes from patients with ulcerative colitis. J Lab Clin. Med *70* 752–759 1967

Hurd E R & Giuliano V J The effect of cyclophosphamide on B- and T-lymphocytes in patients with connective tissue diseases. Arthritis Rheum. *18* 67 75, 1975

H j Lene Jensen F P B., Bonnevie O & Riis P An epidemiological study of regional enteritis and acute ileitis in Copenhagen County Scand J Gastroent. *8* 581 584 1973

Jones, J I Development of delayed hypersensitivity to dinitrochlorobenzene in patients with Crohn disease Gut. *10* 52 56 1969

Kirsner J B & Goldgraber M B Hypersensitivity autoimmunity and the digestive tract. Gastroenterology *38* 536–562, 1960

Kumagai, K Abo T T Sekizawa, T & Sasaki, M Studies of surface immunoglobulins on human B-lymphocytes. I Dissociation of cell-bound immunoglobulins with acid pH or at 37 C J. Immunol *115* 982 987 1975

Lobo P I Westervelt F B & Horwitz D A Identification of two populations of immunoglobulin-bearing lymphocytes in man. J Immunol *114* 116–119 1975.

McFarlan H & Hamid J Cell mediated immune response in malnutrition Clin Exp Immunol *13* 153 164 1973

McHattie J Immunoresponsiveness of lymphocytes from patients with regional ileocolitis (Crohn disease) by in vitro testing. Clin. Res. *19* 779 (Abstr) 1971

Parent K Barrett J & Wilson I D Investigation of the pathogenic mechanisms in regional enteritis with in vitro lymphocyte cultures. Gastroenterology *61* 431–439 1971

Park S K Brody J I., Wallace H A. & Blakemore W S Immunosuppressive effect of surgery Lancet i. 53–55 1971

Pisedo G & Watson D W. Lymphocyte stimulation by colonic and fecal antigens in patients with ulcerative colitis and Crohn's disease. Gastroenterology *60* 706 (Abstr) 1971

Richens E. R. Mixed-lymphocyte reaction as a measure of immunological competence of lymphocytes from patients with Crohn's disease Gut *15* 24–28, 1974

Riddle P R. & Berenbaum M C Postoperative depression of the lymphocyte response to phytohaemagglutinin. Lancet i. 746–748, 1967

Riis, P & Anthonisen P The differential diagnosis between ulcerative colitis and Crohn disease of the colon. In *Gregor O & Riedl, O* (Eds.) VIIIth International Congress of Gastroenterology Prague. Schattauer Stuttgart. New York 1968, pp. 912–913

Röpke C Lymphocyte transformation and delayed hypersensitivity in Crohn disease. Scand. J Gastroent. *7* 671–677 1972.

Sachar D B. Impaired lymphocyte responsiveness in inflammatory bowel disease. Gastroenterology *64* 203–209 1973.

Stobo J D Tomasi, T B., Huizenga K. A., Spencer R J & Shorter R. G In vitro studies of inflammatory bowel disease. Surface receptors of the mononuclear cell required to lyse allogeneic colonic epithelial cells. Gastroenterology *70* 171 176 1976.

Strickland R. G., Husby G Black W C & Williams R C J Peripheral blood and intestinal lymphocyte subpopulations in Crohn's disease Gut *16* 847–853 1975

Strickland R. G Korsmeyer S Soltis R. D., Wilson I D & Williams R. C J Peripheral blood T and B cells in chronic inflammatory bowel disease. Gastroenterology *67* 569–577 1974

Sørensen S F Hardt F & Velen V K. Estimation of lymphocyte subpopulations in the peripheral blood of patients with sarcoidosis. (In press) Scand. J Immunol. 1976.

Thayer W R., Charland C & Field C Salazopyrin and its effect on lymphocyte subpopulations. Gastroenterology *70* A– 84/942 (Abstr) 1976

Walker J G Delayed hypersensitivity and lymphocyte transformation in Crohn disease and proctocolitis. Gut *10* 414 (abstr) 1969

Winchester R J F S M., Hoffman T & Kunkel, H G IgG on lymphocyte surfaces technical problems and the significance of a third cell population J Immunol *114* 1210–1212, 1975

Y D T., Philip J Clements H E., Paulus E., Peter J B., Levy J & Barnett E. I Human

lymphocyte subpopulations effect of corticosteroids. J Clin. Invest. *53* 565–571 1974a.

Yu D T Clements P J Peter J B., Levy J Paulus H E. & Barnett E I .. Lymphocyte characteristics in rheumatic patients and the effect of azathioprine therapy Arthritis Rheum. *17* 37–45 1974b.

Aas J Inflammatory bowel disease lymphocytic responses to non-specific stimulation in vitro. Scand J Gastroent. 7 299–303 1972.

Acta path. microbiol. scand. Sect. C, 85 49–56, 1977

IN VITRO EFFECTS OF RUBELLA VIRUS, STRAIN RA 27/3 ON HUMAN LYMPHOCYTES

I. Viral Inhibition of Mitogen Stimulation in Relation to Rubella Haemagglutination Inhibition Antibodies

ROLF MALLER and LARS SÖRÉN

Department of Clinical Bacteriology and Department of Infectious Diseases, Linköping University Linköping Sweden

Maller R. & Sörén, L. *In vitro* effects of rubella virus strain RA 27/3 on human lymphocytes. Viral inhibition of mitogen stimulation in relation to rubella haemagglutination inhibition antibodies Acta path microbiol. scand. Sect C, 85 49–56, 1977

The inhibiting effect of rubella virus on lymphocyte stimulation *in vitro* was studied, using purified virus of the attenuated strain RA 27/3 Addition of the virus to human lymphocytes from twenty healthy blood donors before stimulation with leuco-agglutinin (LA) a component of phytohaemagglutinin, caused considerable inhibition of the LA response in some experiments, whereas in other experiments the inhibition was slight or non-existent. If further analysed, the results showed a correlation between the degree of inhibition and the immunity of the lymphocyte donor against rubella, as measured by haemagglutination inhibition (HI) Thus the LA-response was significantly more depressed in a group of lymphocyte donors with HI titres ranging from 20 to 160 than in another group with HI-titre less than 5. Possible explanations of the virus-induced inhibition of the LA response and possible connection between this phenomenon and the immune response against rubella are discussed.

Key words Rubella virus lymphocytes in vitro mitogen stimulation haemagglutination inhibition antibodies

R Maller Regionsjukhuset, Linköping S-581 85 Linköping, Sweden.

Received 16 76 Accepted 13 ix 76

Rubella virus infection is known to inhibit the phytohaemagglutinin (PHA) response of lymphocytes cultured *in vitro* (10 19 20 23 24) This was first demonstrated in babies presenting the congenital rubella syndrome (20) and, subsequently the phenomenon has been studied following vaccination against rubella (9 16, 31) Some investigators have reported a normal or only slightly depressed response to PHA (9 11 15 24 34) whereas others have demonstrated a considerable inhibition (16 20, 31) This variation could in part, be explained by differences in the virulence of the rubella strains used (16 23) Addition of live rubella virus to normal lymphocytes *in vitro* inhibits the PHA response of these cells (10 19 23 24) The mechanism involved is not known. The object of the present paper is to study the effect on PHA stimulation of the attenuated rubella virus strain RA 27/3 added to normal human blood lymphocytes *in vitro* and, in particular the differences, if any between rubella im-

mune and non immune lymphocytes with respect to viral inhibition of mitogen stimulation

MATERIAL AND METHODS

Materials Gelatin K 936 was obtained from Edible Compounds Ltd. Hull England. Ficoll was obtained from Pharmacia AB Uppsala, Sweden and sodium metrizoate (Isopaque) from Nyegaard & Co Oslo Norway Tissue culture medium 199 F (TCM 199) obtained from GIBCO Grand Island New York and buffered with HEPES was supplemented with L-glutamine and benzylpenicillin, 1250 IE/ml. Foetal calf serum (Flow Laboratories, Irvine Scotland) and autologous serum were heat inactivated at 56 C for 30 min. Leuco-agglutinin (LA) purified from phytohaemagglutinin (33) from Pharmacia AB Uppsala Sweden, was diluted in physiological saline to different concentrations and added in 0 1 ml aliquots to 0.9 ml of cell suspension. Micro Test II tissue culture plates 3040 (Falcon Plastics, Oxnard, USA) were used for lymphocyte cultures. ^{14}C-TdR, spec.act. 58 mCi/mmole was obtained from The Radiochemical Centre, Amersham England. At harvest cultured cells were collected on glass fibre filters, 934 AH from Reeve Angel, New Jersey USA

Lymphocyte donors Healthy volunteers, thirteen men and seven women aged 20 to 57 years (average 32 years) were the donors of lymphocytes.

Lymphocyte separation Newly drawn venous blood was immediately defibrinated by vigorous shaking with glass pearls and lymphocytes were separated by gelatin sedimentation (5) followed by centrifugation on a layer of Ficoll Isopaque (2) The lymphocytes were washed three times with TCM 199 and were suspended in TCM 199 with 15 per cent foetal calf serum in a conc of 1×10^6 mononuclear cells/ml The final cell suspensions contained about 90 per cent mononuclear cells.

Rubella virus Attenuated rubella virus, strain RA 27/3 was kindly provided by Dr *Pertti Halonen* Orion Diagnostica, Helsinki Finland. The virus was propagated in cultures of BHK 21/13S cells, in total 55 passages, purified by low speed centrifugation followed by ultracentrifugation in a sugar gradient and finally dialysed (29) The cultures were checked for absence of contaminating microorganisms including mycoplasma. The infectivity of the virus preparation was tested by plaque assay on BHK 21/W 3 cells and the titre was adjusted to 10^7 pfu/ml The final product was suspended in 0 025 M phosphate buffer pH 7.3 with EDTA 0 005 M it was rapidly frozen and stored at −70 C. Infectivity was also checked after thawing The virus was transported to Linköping in liquid nitrogen, −196 C and stored after arrival at −90 C. The virus was diluted in 0 025 M phosphate buffer supplemented with EDTA 0 005 M, NaCl 0.15 M and 2 per cent sacharose ("virus buffer") When applicable the virus was UV inactivated by irradiating the virus suspension contained in a quartz vessel for 20 min at 254 nm. The radiation energy obtained in the quartz-vessel was 480 μW/cm^2 Under these conditions rubella virus is inactivated without losing its antigenic properties (7)

HI-test The rubella haemagglutination-inhibiting (HI) antibodies in autologous sera and foetal calf sera were determined by the method of *Cooper et al* (4) Every HI titre figure was set according to the results of three separate determinations. The HI titres of the foetal calf sera used were <5.

Lymphocyte cultures In order to minimize possible reactions between virus and rubella antibodies in the serum of the lymphocyte donor (10) the incubation of lymphocytes together with rubella virus was performed in TCM—15 per cent foetal calf serum in siliconized conical glass tubes. Rubella virus or "virus buffer" in 0 1 ml portions were added to 0 9 ml lymphocyte cultures which were incubated overnight. The cells were spun down, washed twice in TCM 199 and finally resuspended in TCM 199 with 15 per cent autologous serum which proved to be superior in supporting the growth of LA stimulated cells. The mononuclear cell concentration was adjusted to 1×10^6/ml. LA in different concentrations in 0 1 ml aliquots were added to 0 9 ml cultures of virus-infected and non-infected cultures, respectively Four aliquots of 0 2 ml from each specimen were dispensed on microplates which were then closed with a film and incubated at 37 C in humidified air for an indicated number of days. The survival of the cells was checked intermittently using trypan blue exclusion. Five hours before the end of the incubation period, 0 03 μCi of ^{14}C-TdR in 0 μl of saline was added to each culture. Cultures were washed and precipitated onto glass fibre filters by a semiautomatic multiple sample processor (28) The filters were placed in 10 ml Permablend III (Packard Instrument Co., Ill. USA) and the radioactivity was measured in a Packard Tri-Carb 3375 scintillation counter The incorporated radioactivity was expressed as cpm/ml of cell suspension.

Statistics The methodological error of ^{14}C-TdR incorporation and radioactivity determination, standard deviation (SD) was calculated from the values (X) of cultures without virus, stimulated by LA in optimal concentration. An estimated deviation from $\bar{X}$ in a future series of cultures performed under the same conditions (Y) could be prognosticated, using Student's t-distribution. Accordingly the prognosticated interval for $\bar{Y}$ would be

$$\bar{X} \pm t \quad SD_X \sqrt{1/n_X + 1/n_1}$$

Values below and above these limits would be con-

TABLE 1 Results of LA stimulation for Six Days of Lymphocytes from Donors with HI-titre Ranging from 20 to 160

Expt No.	HI titre	Incubation with "virus buffer" cpm/ml × 10[a]	Incubation with 10[4] pfu/ml virus cpm/ml × 10[a]	Inhibition ± %[c]	Incubation with 10[4] pfu/ml virus cpm/ml × 10[a]	Inhibition ± %[c]	Incubation with UV-inact. virus[d] cpm/ml × 10[a]	Inhibition ± %[c]
1	160	16.7	4.6***b	–73	12.4	–27	–	–
2	160	16.9	6.3**	–63	9.5**b	–44	15.6	–8
3	80	19.7	2.4 **	–88	21.9	+11	–	–
4	80	8.9	1.9***	–78	7.8*	–13	8.3	–7
5	80	5.7	2.6**	–55	6.0	+5	6.1	+6
6	80	12.0	8.6	–28	9.2	–23	8.1**b	–33
7	40	10.3	7.3	–29	6.0	+5	8.1	+6
8	40	12.6	12.4	–2	11.8	–8	14.2	+13
9	20	4.9	2.8**	–43	–	–	4.5	–10
Mean ± SD[e]		12.0 ± 5.1	5.4 ± 3.5		11.2 ± 4.8		9.5 ± 4.1	

[a] Thymidine incorporation expressed as cpm/ml of cell suspension. Values approximated to the nearest hundred.

[b] Denotes level of significance of the deviation from mean thymidine incorporation in cultures without rubella virus. * significant at the 1 per cent level and *** significant at the 0.1 per cent level.

[c] Deviation in per cent, i.e. expressed as $(100 \frac{\text{cpm virus}}{\text{cpm control}} - 100)$; (—) before figures denotes inhibition.

[d] Rubella virus 10[4] pfu/ml inactivated by UV irradiation.

[e] SD = standard deviation.

TABLE 2 Results of LA stimulation for Six Days of Lymphocytes from Donors with HI-titre Less than 5

Expt No.	HI titre	Incubation with "virus buffer" cpm/ml × 10[a]	Incubation with 10[4] pfu/ml virus cpm/ml × 10[a]	Inhibition ± %[c]	Incubation with 10[4] pfu/ml virus cpm/ml × 10[a]	Inhibition ± %[c]	Incubation with UV-inact. virus[d] cpm/ml × 10[a]	Inhibition ± %[c]
10	<5	4.9	4.2	–14	–	–	6.8	+39
11		11.6	11.7	+1	13.6	+18	12.8	+11
12		9.6	9.0	–7	9.4	–3	10.2	+6
13		16.8	12.5**b	–26	16.5	–2	16.1	–4
14		16.0	15.4	–4	16.6	+4	18.7	+17
15		14.8	15.1	+2	18.5**b	+25	13.6	–8
Mean ± SD[e]		12.3 ± 4.5	11.4 ± 4.2		14.9 ± 3.5		13.0 ± 4.2	

Symbols and terms are the same as those in Table 1

mune and non immune lymphocytes, with respect to viral inhibition of mitogen stimulation.

MATERIAL AND METHODS

Materials Gelatin K 936 was obtained from Edible Compounds Ltd. Hull England Ficoll was obtained from Pharmacia AB Uppsala, Sweden and sodium metrizoate (Isopaque) from Nyegaard & Co. Oslo Norway Tissue culture medium 199 F (TCM 199) obtained from GIBCO Grand Island, New York and buffered with HEPES was supplemented with L-glutamine and benzylpenicillin 1250 IE/ml. Foetal calf serum (Flow Laboratories, Irvine Scotland) and autologous serum were heat inactivated at 56 C for 30 min. Leuco-agglutinin (LA) purified from phytohaemagglutinin (33) from Pharmacia AB Uppsala, Sweden, was diluted in physiological saline to different concentrations and added in 0 1 ml aliquots to 0.9 ml of cell suspension Micro Test II tissue culture plates 3040 (Falcon Plastics, Oxnard USA) were used for lymphocyte cultures. ^{14}C-TdR spec.act. 58 mCi/mmole was obtained from The Radiochemical Centre Amersham, England. At harvest, cultured cells were collected on glass fibre filters 934 AH from Reeve Angel New Jersey USA

Lymphocyte donors Healthy volunteers, thirteen men and seven women aged 20 to 57 years (average 32 years) were the donors of lymphocytes.

Lymphocyte separation Newly drawn venous blood was immediately defibrinated by vigorous shaking with glass pearls and lymphocytes were separated by gelatin sedimentation (5) followed by centrifugation on a layer of Ficoll-Isopaque (2) The lymphocytes were washed three times with TCM 199 and were suspended in TCM 199 with 15 per cent foetal calf serum in a conc. of 1 × 10^6 mononuclear cells/ml The final cell suspensions contained about 90 per cent mononuclear cells.

Rubella virus Attenuated rubella virus, strain RA 27/3 was kindly provided by Dr *Pertti Halonen* Orion Diagnostica, Helsinki, Finland The virus was propagated in cultures of BHK 21/13S cells, in total 55 passages, purified by low speed centrifugation followed by ultracentrifugation in a sugar gradient and finally dialysed (29) The cultures were checked for absence of contaminating microorganisms including mycoplasma. The infectivity of the virus preparation was tested by plaque assay on BHK 21/W 3 cells and the titre was adjusted to 10^7 pfu/ml The final product was suspended in 0 025 M phosphate buffer pH 7 3 with EDTA 0 003 M it was rapidly frozen and stored at −70 C. Infectivity was also checked after thawing The virus was transported to Linköping in liquid nitrogen, −196 C, and stored after arrival at −90 C. The virus was diluted in 0 025 M phosphate buffer supplemented with EDTA 0 003 M, NaCl 0.15 M and 2 per cent sacharose ("virus buffer") When applicable the virus was UV inactivated by irradiating the virus suspension contained in a quartz-vessel for 20 min at 254 nm. The radiation energy obtained in the quartz-vessel was 480 μW/cm^2 Under these conditions rubella virus is inactivated without losing its antigenic properties (7)

HI-test The rubella haemagglutination-inhibiting (HI) antibodies in autologous sera and foetal calf sera were determined by the method of *Cooper et al* (4) Every HI titre figure was set according to the results of three separate determinations The HI titres of the foetal calf sera used were <3.

Lymphocyte cultures In order to minimize possible reactions between virus and rubella antibodies in the serum of the lymphocyte donor (10) the incubation of lymphocytes together with rubella virus was performed in TCM—15 per cent foetal calf serum in siliconized conical glass tubes. Rubella virus or "virus buffer" in 0 1 ml portions were added to 0.9 ml lymphocyte cultures which were incubated overnight. The cells were spun down, washed twice in TCM 199 and finally resuspended in TCM 199 with 15 per cent autologous serum which proved to be superior in supporting the growth of LA stimulated cells. The mononuclear cell concentration was adjusted to 1 × 10^6/ml. LA in different concentrations in 0 1 ml aliquots were added to 0.9 ml cultures of virus-infected and non-infected cultures, respectively Four aliquots of 0.2 ml from each specimen were dispensed on microplates which were then closed with a film and incubated at 37 C in humidified air for an indicated number of days. The survival of the cells was checked intermittently using trypan blue exclusion. Five hours before the end of the incubation period, 0 05 μCi of ^{14}C-TdR in 20 μl of saline was added to each culture. Cultures were washed and precipitated onto glass fibre filters by a semiautomatic multiple sample processor (28) The filters were placed in 10 ml Permablend III (Packard Instrument Co., Ill., USA) and the radioactivity was measured in a Packard Tri-Carb 3375 scintillation counter The incorporated radioactivity was expressed as cpm/ml of cell suspension.

Statistic The methodological error of ^{14}C TdR incorporation and radioactivity determination, standard deviation (SD) was calculated from the values (X) of cultures without virus, stimulated by LA in optimal concentration. An estimated deviation from $\bar{X}$ in a future series of cultures performed under the same conditions (Y) could be prognosticated using Student's t-distribution Accordingly the prognosticated interval for $\bar{Y}$ would be

$$\bar{X} \pm t \quad SD_X \sqrt{1/n_X + 1/n_Y}$$

Values below and above these limits would be con-

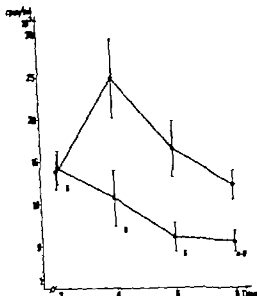


Fig. 3. Time course of thymidine incorporation of lymphocytes incubated with In rubella virus 10^4 pfu/ml (Δ—Δ) or with Virus buffer (○—○). The day following addition of LA to cultures are indicated. Lymphocyte donors with HI-titres from 20 to 160. The results of individual cultures are integrated and shown as mean ± standard error.

with a range of different LA-concentrations. Since the dose-response curves generally are not seen to shift after virus infection, nothing but the thymidine incorporations at optimal LA concentrations are shown in the Tables. The experiments are grouped according to the HI titres of the lymphocyte donors, since these titres are considered to be a good measure of rubella immunity (27). Following the addition of rubella virus in a concentration of 10^4 *pfu/ml* a considerable inhibition of thymidine incorporation was seen in five out of six cases where HI = 80–160 (expt 1–5) while inhibition was moderate in one case where HI = 80 (expt 6). In one out of two cases where HI = 40, a moderate inhibition was observed (expt 7) whereas no inhibition (expt 8) was observed in the other case. In the only case where HI = 20 a moderate inhibition was seen (expt 9). Finally in five out of six cases where serological immunity against rubella was absent, HI<5 the inhibition was nil or slight whereas a moderate inhibition of thymidine incorporation in the sixth case was recorded (expt 13). Altogether these results show that rubella virus-induced inhibition of the LA response is more pronounced in cases where the lymphocyte donor displays immunity against rubella virus. The difference in thymidine incorporation in lymphocyte cultures from donors with definite serological immunity (Table 1) and in lymphocyte cultures from donors without serological immunity (Table 2) was significant ($p<0.025$). This significance was more pronounced ($p<0.01$) when the group with the highest HI titres, i.e. 80–160 was compared with that where HI<5. In general, the inhibition of thymidine incorporation following addition of rubella virus in a concentration of 10 pfu/ml was much less pronounced than that following addition to 10^4 pfu/ml. Addition of UV-inactivated virus did not lead to any significant change in thymidine incorporation as compared with that in control cultures.

3) *Temporal Development of Inhibition of the LA response*

No inhibition of thymidine incorporation was observed on day 3 after addition of LA. However as soon as the LA-stimulation had reached its optimal effect, i.e. from day four on, the inhibition was considerable in the group of lymphocyte donors with HI-titres 20–160 (Fig. 3).

4) *Numbers of Surviving Cells in Virus-infected and Non-infected Cultures*

The number of surviving cells in infected and non-infected cultures (n = 10) were 0.23 ± 0.08 × 10^6/ml and 0.33 ± 0.13 × 10^6/ml, respectively (mean ± SD). These cell counts are subject to great uncertainties because of the cell-clumping effect of LA, but the difference was not significant ($p>0.05$). The percentages of dead cells were also about the same, 29 ± 8 per cent and 26 ± 5 per cent, respectively. Thus, there were no obvious dif

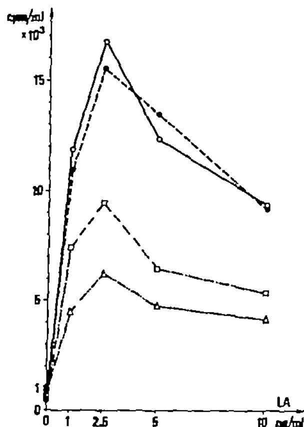


Fig 1 Thymidine incorporation of lymphocytes incubated with live or inactivated rubella virus or with "virus buffer" following 6 days incubation after addition of LA in different concentrations. HI titre of lymphocyte donor = 160 Symbols ○——○ "virus buffer" ●- - -● UV inactivated rubella virus 10^4 pfu/ml Δ- Δ live rubella virus 10^4 pfu/ml and □— —□ live rubella virus 10^3 pfu/ml.

sidered significantly different from ♀. These prognosticated intervals were calculated using t values for p = 0 001 and p = 0 01 respectively Accordingly the differences between control cultures (♀) and virus-treated cultures (♀) were classified as non-significant at the 0 1 and 1 per cent level, respectively Student's t test was used in other statistical calculations.

RESULTS

1) *Pattern of Virus Inhibition of the LA response*

The inhibiting effect of rubella virus strain RA 27/3 on the LA response of lymphocytes varied considerably in different experiments, ranging from no inhibition at all up to almost complete impairment of the LA response (*Table 1 and 2*) *Fig 1* shows one experiment where inhibition of the LA induced thymidine incorporation was pronounced. Evidently the thymidine incorporation into cultures with UV inactivated rubella virus was grossly similar to that of cultures without virus. On the other hand, the incorporation into cultures with rubella virus 10^4 *pfu/ml* was considerably lower than that in control cultures. In cultures with rubella virus 10^3 pfu/ml, a less pronounced inhibition of thymidine incorporation was observed. In this experiment as in *most other experiments, the LA dose response* curves of virus-infected and non-infected cells were quite similar On two occasions, however a depression of the LA response curve applying to virus treated cells was observed at *the optimal LA concentration of non treated* cells (Fig 2)

2) *Inhibition in Relation to HI-titres of the Lymphocyte Donors*

The above pattern of virus induced inhibition of thymidine incorporation was not seen in all experiments. The results of 15 experiments are shown in Tables 1 and 2 In all *these experiments the cells were stimulated*

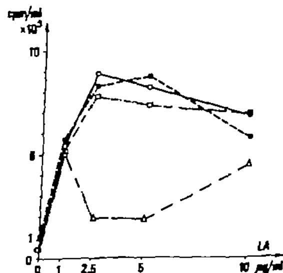


Fig 2 Depression of the LA dose-response curve of lymphocytes incubated with live rubella virus 10^4 pfu/ml Incubation time 6 days after addition of LA. *HI titre of lymphocyte donor* = 80 Symbols as those shown in Fig. 1

3) *Rubella Virus Exerts a Cytotoxic Effect on the Lymphocytes*

According to the present and other studies (10 19) this alternative seems unlikely since any definite increase in cell death in the virus infected cultures could not be observed. On the other hand rubella virus has been shown to replicate in PHA stimulated lymphocytes (5 19) and it is possible that this viral replication might affect the proliferation of the stimulated cells without causing a direct cytotoxic effect.

4) *A Product is Synthesized in the Cultures which Inhibits the Proliferation of LA Stimulated Lymphocytes*

Rubella virus infected cells synthesize poorly characterized products which inhibit the growth of other cells (21) One known inhibitor is interferon which inhibits the proliferation of a number of cells, including PHA stimulated lymphocytes (14) In lymphocyte cultures, interferon is produced by T lymphocytes following immune specific stimulation by viral antigens (6, 22) A proportion of the cells might be expected to be stimulated by rubella virus to produce interferon, and thus, the amount of interferon would increase with time of culture. After some delay this would lead to an inhibition of the LA induced proliferation. Another possible mechanism of the virus induced inhibition is that antibodies, which form inhibiting complexes with rubella virus (12) are produced *in vitro* after immune activation of the lymphocytes by rubella virus Both these hypotheses would possibly explain the apparent correlation between the degree of LA response and serological immunity against rubella. However other factors must also be operative since UV-inactivated virus, which does not inhibit the LA response would also be able to induce both interferon and antibody production *in vitro*

The authors want to thank Mrs. *Ulla Strand* and Miss *Kristin Groth* for skilful technical assistance *Stig Danielson* and *Thomas Larsson* Department of Mathematics, Linköping University for assistance with the statistics. This work was kindly supported by grants from the *Medical Faculty Linköping University* and the *Medical Research Committee of the County Council Östergötlands läns landsting*

REFERENCES

1 *Berkovich S Steiner P & Steiner M*: Live rubella virus vaccine in tuberculosis children. Am. J Dis. Child. *118* 252–257 1969
2 *Bøyum A.*, Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation. Scand. J clin. Lab. Invest Suppl. *97*: 27 50, 1968.
3 *Chappel, R.* Cell mediated immunity in experimental rubella infections. Arch. Virol. *47* 375–379 1975
4 *Cooper L. Z, Matters B., Rosenblum, J K. & Krugman S* Experience with a modified rubella hemagglutination inhibition antibody test. J amer med. Ass. 207 89–93 1969
5. *Coulson A. S & Chalmers G. G.* Separation of viable lymphocytes from human blood. Lancet *i.* 468–469 1964
6 *Epstein L. B., Stevens D A, & Merigan T C* Selective increase in lymphocyte interferon response to vaccinia antigen after revaccination Proc. Nat. Acad. Sci. (USA) *69* 2632–2636, 1972.
7 *Fabiyi A., Sever J L., Ratner N & Caplan B.*: Rubella virus Growth characteristics and stability of infectious virus and complement fixing antigen, Proc. Soc. Exp Biol. Med. *122*: 392 398, 1966.
8. *Honeyman, M C Forrest J M & Dorman, D C.*, Cell-mediated immune response following natural rubella and rubella vaccination. Clin. exp. Immunol. *17* 665–671 1974
9 *Kauffman C A Phai J P Linnemann C C & Schiff G. M.*, Cell mediated immunity in humans during viral infection. Infection and Immunity *10* 212–215 1974
10. *Knight S C & Najera R.*: The effect of rubella virus on the growth of lymphoma cells and normal lymphocytes in vitro. Microbios *2*: 185–191 1969
11 *Lalla M Vesikari T & Virolainen M* Lymphoblast proliferation and humoral antibody response after rubella vaccination. Clin. exp. Immunol. *15* 193–202, 1973.
12. *Lee J C. & Sigel M M.* A differential effect of IgM and IgG antibodies on the blastogenic response of lymphocytes to rubella virus. Cell. Immunol. *13*: 22 31 1974
13 *Levis W R & Robbins J H.*, Effect of glass-adherent cells on the blastogenic response of purified lymphocytes to phytohemagglutinin. Expl. Cell. Res. *61* 153–158, 1970.

ferences between infected and non infected cells with regard to number of cells.

DISCUSSION

Both humoral (30) and cellular immunity develop after infection with rubella virus. Cell mediated immunity as demonstrated by lymphocyte cytotoxicity against rubella infected cells (26) development of cytotoxic lymphokines (32) MIF production (8) and antigen specific lymphocyte stimulation (24 31 32) have been shown to coincide with or precede the development of humoral antibodies. Parallel to the development of specific immunity against rubella virus, a non specific anergy arises, as revealed by an impairment of cellular immune reactions against various antigens and mitogens. This is demonstrated by a decreased dermal hypersensitivity against streptokinase streptodornase (9) and possibly against tuberculin (1) as well as by an inhibition of PHA induced lymphocyte stimulation (16 17 31 32) This anergy is seen in many viral infections (18) and its mechanism and significance are still obscure.

Studies of the PHA response of lymphocytes from vaccines given different attenuated strains of rubella virus have yielded variable results. Some investigators have reported a normal or only slightly reduced response to PHA (9 11 17) whereas others have demonstrated a considerable depression (16 31 32) Inhibition induced by attenuated virus strains is generally less intense than that induced by virulent strains (16 23) Addition of rubella virus to *in vitro* cultures of normal lymphocytes inhibits the PHA stimulation of these cells, provided that the virus is added before or simultaneous with the PHA (10 19 23 24) UV inactivation (10, 19 24) and treatment of the virus with anti rubella antibodies abolish the inhibition (10) However the effect of antibodies is complex, since recent results show that IgM anti rubella antibodies cooperate with rubella virus in the inhibition of PHA response (12)

In the present study a variable degree of inhibition of the LA response was in different experiments seen to follow incubation of human lymphocytes with the attenuated purified rubella virus strain RA 27/3 In the experiments, where inhibition occurred the thymidine incorporation into infected and non-infected cultures was similar up to the third day of culture with LA, i.e there was an initial time interval before the inhibition was fully developed. The most pronounced inhibition was observed in experiments using lymphocyte from individuals with high titres of anti rubella HI-antibody whereas the inhibition in experiments using lymphocytes from sero-negative donors was slight or nonexistent. Although no significant correlation between the level of HI titre and the degree of inhibition could be obtained, the mean thymidine incorporation of rubella virus treated lymphocytes from donors with definite serological immunity was significantly lower than that of lymphocytes from non-immune donors The mechanism involved in the inhibition of the LA response of lymphocytes induced by rubella virus is still uncertain. At least four explanations can be put forward

1) *The Rubella Virus Interferes Directly with the LA Stimulation Process*

This alternative seems less likely since, as a rule, no inhibition was observed during the first three days of culture Furthermore, there was no displacement of the dose response curve for LA, which might be expected if the virus for example interfered with the binding of LA to the lymphocytes.

2) *Monocytes Are Infected by Rubella Vir*

Monocytes augment the proliferative re sponse of PHA stimulated lymphocytes (13 Polio virus has been shown to infect mono cytes in leukocyte cultures and thereby abolis the augmenting effect of these cells in PH stimulation (25) In the rubella system how ever the viral inhibition of the PHA respons of cultures with and without glass adheren cells has not been found to differ (31)

Acta path. microbiol. scand. Sect. C, 85: 57–64 1977

IMMUNE COMPLEXES IN CYSTIC FIBROSIS

PETER OLUF SCHIØTZ, NIELS HØIBY, FREDE JUHL, HENRIK PERMIN
HENRIK NIELSEN and SVEN ERIK SVEHAG

The Paediatric Clinic TG Rigshospitalet, the Immunological Laboratory University Clinic for Infectious Diseases, Rigshospitalet, Statens Seruminstitut, Department of Clinical Microbiology at Hvidovre Hospital, Copenhagen and the University Institute for Medical Microbiology Odense Denmark

Schiøtz, P. O., Høiby, N., Juhl, F., Permin, H., Nielsen, H. & Svehag, S. E. Immune complexes in cystic fibrosis. Acta path. microbiol. scand. Sect. C, 85: 57–64 1977

Eleven patients with cystic fibrosis (CF) chronically infected with mucoid *P. aeruginosa* and ten patients without *P. aeruginosa* infection were examined for occurrence of circulating immune complexes, for immune complex deposits in the dermo-epidermal junction of the skin and for precipitins against *P. aeruginosa*, *S. aureus*, *H. influenzae* and *D. pneumoniae* antigens. The serum concentrations of haptoglobin, orosomucoid, immunoglobulins, C1q, C3, C4 and total haemolytic complement, antinuclear and rheumatoid factor activities as well as white blood cell counts and erythrocyte sedimentation rates were determined also. The results indicated that 6 patients from the chronically *P. aeruginosa* infected group, exhibiting a spectrum of serum precipitins against *P. aeruginosa* antigens, also had immune complexes in the serum, while only one patient (suffering from selective IgA deficiency) in the group without *P. aeruginosa* infection was positive for soluble immune complexes. Granular deposits of IgM was found in the skin of 10 of the chronically *P. aeruginosa* infected patients and in 7 of the patients without *P. aeruginosa* infection. A few patients in both groups had dermo-epidermal deposits of C1q, C3 or fibrinogen as well. Eight of the patients in the chronically infected group and five in the group without *P. aeruginosa* infection had organ non-specific antinuclear factors. The haptoglobin levels appeared to be elevated in the chronically infected patients ($p<0.05$). None of the other parameters showed any significant difference between the two groups.

Key words: Cystic fibrosis; immune complexes; *Pseudomonas aeruginosa*; skin biopsy; complement; antinuclear factor

P. O. Schiøtz, Paediatric Department TG, Rigshospitalet, Tagensvej 18, DK 2200 Copenhagen N, Denmark

Received 21 76 Accepted 14.ix.76

Immune complexes have recently been demonstrated (21) in necropsy specimens from patients with cystic fibrosis (CF). Deposits of complexes were indicated in several organs such as spleen, thymus, liver, stomach, duodenum, pancreas, lung and trachea (21). The origin of these immune complexes is not known. However as CF patients suffer from chronic and recurrent bacterial infections, complex formation between soluble bacterial antigens and antibacterial antibodies may contribute to the tissue damage in the lungs (6).

The primary purpose of the present study was to investigate the occurrence of circulating immune complexes in the blood and of deposited complexes in the skin of CF patients.

Another object of the study was to examine

14 *Lindahl Magnusson P Leary P & Gresser I* Interferon inhibits DNA synthesis induced in mouse lymphocytes suspensions by phytohaemagglutinin or by allogeneic cells. Nature (New Biol) 237 120–121 1972.

15 *Marshall W C Cope W A Soothill J F & Dudgeon J A* In vitro lymphocyte response in some immunity deficiency diseases and intrauterine virus infections. Proc. Roy Soc. Med. *63* 351–354 1970

16. *McMorrow L E Vesikari T Wolman S R Giles J P & Cooper L Z* Suppression of the response of lymphocytes to phytohemagglutinin in rubella. J Infect. Dis. *130* 464–469 1974

17 *Midulla M., Businco L & Moschini L* Some effects of rubella vaccination on immunologic responsiveness. Acta Ped. Scand. *61* 609–611 1972

18. *Notkins A L Mergenhagen S E & Howard R J* Effect of virus infections on the function of the immune system. Ann Rev Microbiol. *24* 525–538 1970

19 *Olson G B Dent P B Rawls W E South M A Montgomery J R Melnick J L & Good R A* Abnormalities of in vitro lymphocyte responses during rubella virus infections. J Exp Med. *128* 47–68 1968.

20 *Olson G B South M A & Good R A* Phytohemagglutinin unresponsiveness of lymphocytes from babies with congenital rubella Nature *214* 695–696 1967

21 *Plotkin S A & Vaheri A* Human fibroblasts infected with rubella virus produce a growth inhibitor Science *156* 659–661 1967

22 *Rasmussen L E Jordan G W Stevens D A & Merigan T C* Lymphocyte interferon production and transformation after herpes simplex infections in humans. J Immunol. *112* 728–735 1974

23 *Savel H & Phillips C A* Decreased inhibition of phytohemagglutinin-induced lymphocyte transformation by attenuated strains of rubella virus. Microbios *5* 49–52 1970

24 *Simons M J & Fitzgerald M G* Rubella virus and human lymphocytes in culture Lancet *ii* 937–940 1968.

25 *Soontiëns F J C J & van der Veen J* Evidence for a macrophage mediated effect of polio virus on the lymphocyte response to phytohemagglutinin. J Immunol. *111* 1411 1419 1973

26 *Steele R W., Hensen S A Vincent M M., Fucillo D A & Bellanti J A.* Development of specific cellular and humoral immune responses in children immunized with the rubella virus vaccine J Infect. Dis. *130* 449–453, 1974

27 *Stewart G L Parkman P D Hopps H E, Douglas R D Hamilton J P & Meyer H M Jr* Rubella virus hemagglutination-inhibition test. New Engl J Med. *276* 554–557 1967

28. *Thurman G B., Strong D M Ahmed A Green S S Sell K W Hartzman R J & Bach F H* Human mixed lymphocyte cultures. Evaluation of a microculture technique utilizing the multiple automated sample harvester (MASH) Clin. exp Immunol. *15* 289–302, 1973

29 *Vaheri A von Bonsdorff C-H., Vesikari T Hovi T & Väänänen P* Purification of rubella virus particles. J gen. Virol *5* 39–46, 1969

30 *Vesikari T* Immune response in rubella infection. Scand. J Infect. Dis. Suppl. *4* 11–1?, 197?

31 *Vesikari T & Buimovici-Klein E.* Lymphocyte responses to rubella antigen and phytohemagglutinin after administration of the *Ra 27/3 strain of live attenuated rubella vaccine.* Infection and Immunity *11* 748–753 1975

32. *Vesikari T Kanra G Y Buimovici-Klein E & Cooper L Z* Cell-mediated immunity in rubella assayed by cytotoxicity of supernatants from rubella virus-stimulated human lymphocyte cultures. Clin. exp. Immunol *19* 33–45 1975

33 *Weber T H* Isolation and characterization of a lymphocyte-stimulating leucoagglutinin from red kidney beans (*Phaseolus vulgaris*) Scand. J clin. Lab Invest. Suppl *111* 33–46, 1969

34 *Wolman S R McMorrow L E Zirra? P R & Cooper L Z* Lack of chromosomal breakage in congenital rubella. Pediatrics *52* 213–2?0 1973

TABLE 1 *Leucocyte Counts, Erythrocyte Sedimentation Rates (ESR) and Concentrations of Immunoglobulins and two Acute Phase Proteins in Blood from Cystic Fibrosis Patients with (CF+P) and without (CF−P)* Pseudomonas aeruginosa *Infection*

	Orosomucoid (g/l)	Haptoglobin (μmol/l)	ESR (mm)	Leucocyte count (× 10⁹/l)	IgG (g/l)	IgA (g/l)	IgM (g/l)
rmal values	(0.50–1.17)	(3–22)	(0–8)	(3.5–7.0)	(6.82–15.7)	(0.56–3.30)	(0.18–1.29)
+P mean	1.43	27.4	11	7.3	19.2	2.37	1.01
range	(0.83–2.03)	(8–52)	(1–30)	(4.8–11.8)	(10.7–32.8)	(0.97–4.54)	(0.62–1.50)
P mean	1.05	17.4	6	5.9	13.6	1.77	0.79
range	(0.69–1.79)	(3–39)	(1–14)	(3.7–8.3)	(7.6–20.6)	(0–3.34)	(0.44–1.13)
gnificance of difference	n.s.	p<0.05	n.s.	n.s.	s.	n.s.	n.s.

NHS devoid of anti-*P. aeruginosa* antibodies, caused no complement consumption in the assay. The SD$_w$ for the assay was 3.4 per cent and the SD$_b$ was 1.9 per cent.

Antinuclear and Rheumatoid Factors in Serum

Sera from all patients were examined for the occurrence and titre of IgG, IgM, IgA and complement C3 fixing granulocyte specific antinuclear factors (GS-ANF) and organ-nonspecific antinuclear factors (ON-ANF) as reported previously (31, 32). Rat liver cryostat sections and smears of isolated and washed human leucocytes served as nuclear substrates.

Rheumatoid factors were demonstrated by the RA-latex fixation slide test (Behringwerke, West Germany). Titres≧32 were considered pathological.

Skin Biopsies

Biopsies from apparently normal skin were obtained from the extensor side of the forearm by punch biopsy technique (4 mm). The skin was anaesthetized with ethyl chloride spray. The tissue was immediately frozen and stored at −70° C. 4–8 μm thick sections were cut in a cryostat within one week after biopsy. The sections were air-dried for 15 minutes, washed in PBS for 30 minutes and incubated with one drop of diluted conjugate in a moist chamber for 30 minutes. Blocking with unconjugated antisera was included as control. The fluorescein isothiocyanate (FITC)-labelled rabbit IgG preparations specific for human α, μ and γ chains, the β1 component of human C3, and fibrinogen, respectively, were obtained from Dakopatts, Copenhagen. FITC-labelled antihuman C4 conjugate and rabbit antisera specific for human IgD, IgE and the C1q component of human complement were obtained from Behringwerke (Marburg, West Germany). The isolated IgG fraction from these latter antisera were labelled with FITC as described earlier (31).

The conjugates against the 5 human immunoglobulins were tested for specificity on monoclonal bone marrow specimens from patients with myelomas and macroglobulinaemia (31). The *P. aeruginosa* standard antibody was labelled with FITC as previously described (14). All conjugates showed monospecific reactions to the corresponding antigens in crossed immunoelectrophoresis (31) and the fluorescein/protein ratios as estimated by OD 495/280 nm were 0.5–0.7. No nonspecific staining at the working dilution was seen, and skin biopsies examined with the different conjugates from 25 normal adults were all negative. The sections were examined in a Leitz Orthoplan fluorescence microscope equipped for incident light illumination using an Osram HBO 200 mercury lamp as light source, a 4 mm BG 38 glass filter, two KP 490 interference filters and a TK 485 dichroic mirror for selection of excitation light, and 1.5 mm 530 nm glass filter served as barrier filter.

Statistical Methods

The Mann-Whitney test and Fisher's test were used and a significance level of 5 per cent (double tailed test) was chosen (3).

RESULTS

The number of specific precipitins to soluble *P. aeruginosa* antigens in the CF+P group averaged 26 (range 12–46) while virtually no precipitating antibodies to these antigens were observed in the CF−P group (average no. of precipitins 0, range 0–2). It applies to sera from all the 21 patients that a limited number of specific precipitins to the anti-

whether the presence of immune complexes could be correlated to the presence of chronic *P aeruginosa* lung infection which has been shown to be associated with a pronounced humoral immune response and a poor prognosis (8 25)

PATIENTS AND METHODS

Patients

(i) This group consisted of 11 CF patients (7 males, 4 females, mean age 13 years, range 9–23) suffering from chronic infection with mucoid *P aeruginosa* (CF+P) and exhibiting more than 10 different precipitins in serum against water soluble antigens from this bacterium.

The patients have been followed as previously described (6 7) Mean duration of the *P aeruginosa* respiratory tract infection was 3.5 years (range 1–6 years) Routine studies of the lung function showed a reduced vital capacity and peak expiratory flow rate as compared with normal values (28) On the average the vital capacity of the lungs was 1 1 SD below mean normal values, and the peak expiratory flow rate was 0 7 SD below normal values in healthy persons of the same height.

(ii) Ten CF patients (6 males 4 females, mean age 13 years, range 8–21) without *P aeruginosa* infection (CF−P) and without multiple serum precipitins against *P aeruginosa* antigens were examined also The average vital capacity of their lungs was 0 6 SD below mean normal values, and the peak expiratory flow rate was 0 1 SD below mean normal values (28)

There were no significant differences between the CF+P and the CF−P groups as regards mean weight and mean height of patients and, in these respects, the values in the 2 groups were less than 1 SD below the normal values in healthy persons of the same age.

In nine of the CF−P patients *S aureus H influenzae K ozaenae* or *E coli* were isolated from the respiratory tract during this study

Antibacterial Precipitins

The occurrence of circulating precipitating antibodies against *P aeruginosa S aureus H influenzae* and *D pneumoniae* was examined by crossed immunoelectrophoresis (8) The bacterial antigens were water soluble extracts obtained by sonication of whole bacterial cells as described previously (6 10) The bacteria used were *P aeruginosa* soluble antigen concentration 11.8 g per litre *D pneumoniae* type 23F soluble antigen concentration 4 8 g per litre a non capsulated *H influenzae* soluble antigen concentration 6.4 g per litre and finally 4 *S aureus* strains representing the 4 phage groups, antigen concentration 8 6 g per litre (5)

Immunoglobulins Acute Phase Proteins Complement Factors and Leucocyte Counts

The concentration in serum of IgG IgA, IgM, IgD haptoglobin, orosomucoid, C1q, C3 and C4 were determined by means of routine electroimmunoassays (17) using monospecific rabbit antibodies (Dakopatts, Copenhagen) against these serum proteins and Standard Human Serum from Behringwerke (Marburg, West Germany) as standard. The concentration of IgE in serum was assessed by the radio-immunosorbent test (Phadebas IgE test, Pharmacia Uppsala Sweden) The results are given as units per millilitre. One unit is approximately 2 4 ngs. The concentration of IgD in serum is given in arbitrary units. 1 arb. U = 1/100 of British Research Standard number 67/37 The concentrations of C1q and C4 are also given as arbitrary units, determined on the basis of a pool of normal plasma—giving 100 arbitrary units per litre as average. Total haemolytic complement levels in serum were determined by measuring the amount of fresh serum required for 50 per cent lysis of sensitized erythrocytes employing the method described by *Mayer* (20) Leucocyte count determinations were performed routinely by a Coulter S automatic electronic counter

Immune Complexes in Serum

From each patient, 5 ml blood was drawn and allowed to coagulate at 37 C for 1 hour The serum was frozen to −70 C within 2 hours and stored at this temperature until it was analysed A complement consumption assay (23) was used for determination of circulating immune complexes. The serum sample (150 µl) was heated at 56° C for 30 min (23) mixed with 20 µl absorbed guinea pig serum and incubated at 37 C for 30 min. The mixture was titrated in twofold dilution in veronal buffer pH 7 4 100 µl of a 4 per cent suspension of sensitized (32 haemolysing units) and washed sheep erythrocytes were added to each tube the tubes were shaken and further incubated at 37 C for 30 min. One ml cold phosphate buffered saline, pH 7 2 (PBS) was pipetted into each tube the tubes were centrifuged at 3000 × g for 5 min and the haemoglobin release was determined spectrophotometrically at 545 nm Veronal buffer was used as a reference CH_{50} and the anticomplementary activity of the specimen tested was expressed as per cent inhibition of the CH_{50} release. Human IgG (20 µg/ml) aggregated at 63 C for 1? min and preformed bovine serum albumin-antialbumin complexes were used as positive controls and normal human sera (NHS) free of heterophilic antibodies to the target cells, as negative controls

Furthermore *P aeruginosa* antigens added to

TABLE 1 *Leucocyte Counts Erythrocyte Sedimentation Rates (ESR) and Concentrations of Immunoglobulins and two Acute Phase Proteins in Blood from Cystic Fibrosis Patients with (CF+P) and without (CF−P)* Pseudomonas aeruginosa *I f ction*

	Orosomucoid (g/l)	Haptoglobin (μmol/l)	ESR (mm)	Leucocyte count (× 10⁹/l)	IgG (g/l)	IgA (g/l)	IgM (g/l)
mal alues	(0.50–1 17)	(3–22)	(0–8)	(3.5–7.0)	(6.82 15 7)	(0.56–3.50)	(0.18–1.29)
+P mean	1 43	27 4	11	7.5	19 2	2.37	1.01
range	(0 85–2.05)	(6–52)	(1–30)	(4.8–11 8)	(10 7–32 8)	(0 97–4 54)	(0.62 1.50)
P mean	1.05	17 4	6	5.9	13.6	1 77	0.79
range	(0.69–1 79)	(5–39)	(1 14)	(3 7–8.7)	(7.6–20 6)	(0–3.54)	(0.44–1 15)
ificance of ference	n.s.	$p<0.05$	n.s.	n.s.	n.s.	n.s.	n.s.

NHS devoid of anti-*P aeruginosa* antibodies, caused no complement consumption in the assay. The SD$_w$ for the assay was 3 4 per cent and the SD$_b$ was 1.9 per cent.

Antinuclear and Rheumatoid Factors in Serum

Sera from all patients were examined for the occurrence and titre of IgG IgM, IgA and complement C3 fixing granulocyte specific antinuclear factors (GS-ANF) and organ-nonspecific antinuclear factors (ON-ANF) as reported previously (31 32). Rat liver cryostat sections and smears of isolated and washed human leucocytes served as nuclear substrates.

Rheumatoid factors were demonstrated by the RA-latex fixation slide test (Behringwerke West Germany) Titres≧32 were considered pathological.

Skin Biopsies

Biopsies from apparently normal skin were obtained from the extensor side of the forearm by punch biopsy technique (4 mm) The skin was anaesthetized with ethyl chloride spray The tissue was immediately frozen and stored at −70 C. 4 8 μm thick sections were cut in cryostat within one week after biopsy The sections were air-dried for 15 minutes, washed in PBS for 30 minutes and incubated w th one drop of diluted conjugate in a moist chamber for 30 minutes. Blocking with unconjugated antisera was included as control. The fluorescein isothiocyanate (FITC)-labelled rabbit IgG preparations specific for human α μ and γ hains, the β1c component of human C3 and fibrinogen, respectively were obtained from Dakopatts, Copenhagen FITC-labelled antihuman C4 conjugate and rabbit antisera specific for huma IgD, IgE and the C1q component of human complement were obtained from Behringwerke (Mar burg West Germany) The isolated IgG fraction from these latter antisera were labelled with FITC as described earlier (31)

The conjugates against the 3 human immunoglobulins were tested for specificity on monoclonal bone marrow specimens from patients with myelomas and macroglobulinaemia (31) The *P aerugi nosa* standard antibody was labelled with FITC as previously described (14) All conjugates showed monospecific reactions to the corresponding antigens in crossed immunoelectrophoresis (31) and the fluorescein/protein ratios as estimated by OD 495/280 nm were 0.5–0.7 No nonspecific staining at the working dilution was seen, and skin biopsies examined with the different conjugates from 25 normal adults were all negative. The sections were examined in a Leitz Orthoplan fluorescence microscope equipped for incident light illumination using an Osram HBO 200 mercury lamp as light source, a 4 mm BG 38 glass filter two KP 490 interference filters and a TK 485 dichroic mirror for selection of excitation light, and a 1.5 mm 530 nm glass filter served as barrier filter

Statistical Methods

The Mann-Whitney test and Fisher's test were used and a significance level of 5 per cent (double tailed test) was chosen (3)

RESULTS

The number of specific precipitins to soluble *P aeruginosa* antigens in the *CF+P* group averaged 26 (range 12-46) while virtually no precipitating antibodies to these antigens were observed in the CF−P group (average no. of precipitins 0, range 0-2) It applies to sera from all the 21 patients that a limited number of specific precipitins to the anti-

TABLE 2 *Concentration of Immunoglobulin IgD IgE and Complement Factors C1q C3 and C4 and Total Haemolytic Complement in Blood from Cystic Fibrosis Patients with (CF+P) and without (CF-P)* Pseudomonas aeruginosa *Infection*

	IgD (arb units/l)	IgE (× 10³ units/l)	C1q (arb. units/l)	C3 (g/l)	C4 (arb. units/l)	Total haemolytic complement (CH₅₀ units/ml)
Normal values	(0 50)	(?3–615)	(68–134)	(0.37–0 79)	(40–135)	(77–112)
CF + P mean	10	89	105	0 74	88	83
range	(0–27)	(29–306)	(9?–123)	(0 61–0.91)	(64–117)	(4?–104)
CF − P mean	19	107	103	0 74	101	85
range	(3–54)	(21–244)	(83–132)	(0 60–0 88)	(66–187)	(60–118)
Significance of difference	n s.	n.s.	n.	n.s.	n.s.	n.s

gens of *S aureus* (average 2.1 range 0–6) *H influenzae* (average 3 0 range 0–6) and *D pneumoniae* (average 1 4 range 0–4) was observed. In this respect there was no significant difference between the CF+P group and the CF−P group

In Table 1 the values of orosomucoid, haptoglobin, IgG IgA, IgM erythrocyte sedimentation rates (ESR) and leucocyte counts are shown If the CF+P and the CF−P groups were compared only the haptoglobin concentrations appeared to differ significantly No significant difference between the two groups was observed as regards IgD IgE and complement factors C1q C3 C4 levels and total haemolytic complement (Table 2)

TABLE 3 *Occurrence of Immune Complexes in Serum from Cystic Fibrosis Patients with (CF+P) and without (CF−P)* Pseudomonas aeruginosa *Infection*

	Positive*/Total
CF + P	6/11
CF − P	1/10
Significance of difference	$0.05 < p < 0.10$

* Inhibition in the complement consumption assay at the 95 per cent confidence level.

Table 3 shows that 6 patients in the CF + P group had circulating immune complexes while complexes were indicated in only one patient in the CF−P group This difference is only significant at the 10 per cent level.

TABLE 4 *Skin Biopsies from Cystic Fibrosis Patients with (CF+P) and without (CF−P)* Pseudomonas aeruginosa *Infection Examined for Deposits of IgG IgA IgM IgD IgE C1q C3 C4 Fibrinogen and Antigens from* Pseudomonas aeruginosa

	CF + P 11 patients	CF − P 10 patients
Number positive for IgG IgA IgD IgE, C4 and *P aeruginosa*	0	0
IgM	10	7
C1q	1	0
C3	2	1
Fibrinogen	1	1

TABLE 5 *Organ non-Specific Antinuclear Factor (ON ANF) of the IgG IgA IgM and C3-fixing Classes in Serum of Cystic Fibrosis Patients with (CF+P) and without (CF−P)* Pseudomonas aeruginosa *Infection*

	ON ANF IgG	ON ANF IgA	ON ANF IgM	ON-ANF C3
CF + P 11 patients	3	?	5	0
CF − P 10 patients	2	2	1	1
Total	5(24%)	4(20%)	6(29%)	1(5%)



Fig 1 Deposits of IgM in granular pattern in the dermo-epidermal junction zone

The patient in the CF−P group who had circulating immune complexes, however differed from the remaining patients in this study in that he was found to present a selective IgA deficiency (IgA ≈ 0 g/l) In addition, precipitating antibodies to two soluble *P aeruginosa* antigens were demonstrable in his serum while no precipitins to *P aeruginosa* antigens were detected in the other patients in the CF−P group If this patient be excluded from the calculations, the difference between the CF+P group and the CF−P group will be significant ($p<0.05$) as far as the occurrence of circulating immune complexes is concerned.

In the skin biopsies we found deposits of immunoglobulin M of a granular pattern at the dermo-epidermal junction in 17 of the 21 patients (Fig 1) Ten of these patients belonged to the CF+P group (10/11) and 7 to the CF−P group (7/10) (Table 4)

Complement C1q and C3 components as well as fibrinogen were found in the skin from patients belonging to both groups, but less frequently (Table 4) The latter patients all exhibited the granular dermo-epidermal deposits of IgM. Fixation of other immunoglobulin classes or of *P aeruginosa* antigens was not demonstrable in the skin biopsy materials from any patient.

GS-ANF or rheumatoid factor assays were negative in all serum samples tested. In contrast, ON ANF was found in both groups of patients (Table 5) A total of 13 patients had positive ON ANF reactions of at least one of the four (IgG IgA, IgM and C3) types. Eight patients belonged in the CF+P group and 5 patients in the CF−P group The number of positive ON-ANF reactions of the IgM type was 5 times as high in the CF+P group as in the CF−P group. Only 3 patients had positive ON ANF of 2 types and no patient had more than two

DISCUSSION

This study indicates the *in vivo* formation of soluble immune complexes in patients with CF particularly in those suffering from chronic *P aeruginosa* infections of the lungs. The complement consumption assay used for immune complex detection in the present study has been applied earlier to model systems (23) as well as to patient sera of various categories (24) Owing to the sensitivity of the assay complexes as small as 10 μg preformed immune complexes/ml NHS may be detected complexes of intermediate as well as large (>19S) size are demonstrable, but immune complexes formed in slight to moderate antibody excess are most effectively detected by this method (23) A source of error in this assay as in other methods for immune complex detection, is aggregated IgG produced by handling and storage of the serum samples. However it is unlikely that this effect has influenced the results reported here as NHS treated in the same way as the patient sera showed ≤5 per cent positive reactions while the CF+P patient group de

monstrated 55 per cent (23–83 per cent with 95 per cent confidence) positiveness.

The origin of the soluble immune complexes is unknown. However localized type III hypersensitivity reactions in the lungs due to complex formation between *P aeruginosa* antigens and the large number of specific precipitins formed to these antigens have been proposed earlier as a possible mechanism participating in the tissue destruction (6 9 25) This hypothesis finds support in experimental studies demonstrating immune complex induced lung damage (4 12 19) Dependent on the antigen/antibody ratio small soluble recirculating immune complexes, or preferably larger complexes will be formed the latter may remain in the lung tissue, resulting in a type III reaction (18) or they are rapidly cleared from the circulation.

It is known that Ig is found in the dermo-epidermal junction in clinically normal skin from patients with systemic lupus erythematosus and that these patients have circulating immune complexes (1 16 29) In the present study granular deposits of IgM as well as of C1q C3 and fibrinogen were observed in both groups of patients although they were most frequent in skin biopsies from patients in the CF+P group The inability to detect deposited *P aeruginosa* antigens in the skin biopsies from patients in the CF+P group may either be due to blocking by IgM and C-factors or to a restricted multispecificity of the antibody preparation used.

It is noteworthy that *P aeruginosa* antigens have been detected previously in serum and sputum from CF+P patients (6 25) Furthermore one of our CF patients with chronic *P aeruginosa* infection and strong precipitin response against soluble *P aeruginosa* antigens as well as two other CF patients with chronic *H influenzae* infections and a spectrum of precipitins against *H influenzae* antigens have developed circulating cryoglobulins considered to represent immune complexes (22)

A possible complement consumption *in vivo* by immune complexes was not reflected by changed concentrations of total haemolytic complement or of the complement components under study Future studies should include investigations of the occurrence of cleavage products of complement factors to determine whether complement activation occurs in CF patients (13)

In accordance with previous results (11) most of the laboratory values reflecting inflammatory reactions and humoral immune responses were not significantly different in the CF+P group and the CF−P group but increased as compared with normal values The negative RA latex fixation test in all patients is in agreement with previous results which showed that RF of the IgM class is infrequent in CF patients (10)

The occurrence of IgG ON ANF in 24 per cent of the patients also corresponds with earlier findings (10) and is in accordance with the situation in patients with other prolonged respiratory diseases accompanied by tissue destruction (2 18, 30) Antinuclear antibodies of certain specificities (anti DNA, anti DNA histone) are known to participate in reactions of the immune complex type *in vivo* (15 26 27) The antibody specificities responsible for the ON ANF reactions observed in sera from some of the CF patients should be examined further

---

This work was supported by the *Thorvald Madsen Legat* the *Danish Medical Research Council*, the *National Danish Association against Cystic Fibrosis* the *Hindsgaul Foundation* and the *National Association against Rheumatic Diseases*.

We thank *Anni Bethien Lis Christiansen* and *George Fam* for skilful technical assistance the Department of Clinical Chemistry and Dr *B. Weeke* Rigshospitalet, Copenhagen for determination of plasma protein concentrations and Statens Seruminstitut, Copenhagen for total complement determinations.

REFERENCES

1 *Baart de la Faille-Kuyper E H Lupus erythematosus. An immunohistochemical and clinical study of 485 patients* M D Thesis, Utrecht, 1969

2 *Bonomo L Gillardi U & Turn A* Anti-*a-globulin and antinuclear factors in chronic*

bronchitis. Amer. J. Clin. Path. 45 315–322, 1966.

3. Diem A. (ed.) *Documenta Geigy Scientific Tables.* J. R. Geigy S. A. Basel 1962, p. 109–127

4. *Gordon D. S., Hunter R. G. O'Reilly R. J. & Conway B. P.* *Pseudomonas aeruginosa* allergy and humoral antibody mediated hypersensitivity pneumonia. Amer. Rev. Resp. Dis. *108* 127–131 1973

5. *Hoff Gerd E. & Høiby N.* *Staphylococcus aureus* in cystic fibrosis antibiotic sensitivity and phage types during the latest decade. Investigations of the occurrence of protein A and some other properties of recently isolated strains in relation to the occurrence of precipitating antibodies. Acta path. microbiol. scand. Sect. B *83* 219–225 1975

6. *Høiby N. & Axelsen N. H.* Identification and quantitation of precipitins against *Pseudomonas aeruginosa* in patients with cystic fibrosis by means of crossed immunoelectrophoresis with intermediate gel. Acta path. microbiol. scand. Sect. B *81* 298–308 1973

7. *Høiby N.* Epidemiological investigations of the respiratory tract bacteriology in patients with cystic fibrosis. Acta path. microbiol. scand. Sect. B, *82* 541 550 1974

8. *Høiby N.* *Pseudomonas aeruginosa* infection in cystic fibrosis. Relationship between mucoid strains of *Pseudomonas aeruginosa* and the humoral immune response. Acta path. microbiol. scand. Sect. B, *82* 551–558, 1974

9. *Høiby N. Jacobsen L., Jørgensen B. A. Lykkegård E. & Weeke B.* *Pseudomonas aeruginosa* infection in cystic fibrosis. Occurrence of precipitating antibodies against *Pseudomonas aeruginosa* in relation to the concentration of sixteen serum proteins and the clinical and radiographical status of the lungs. Acta paediat. scand. *63* 843–848 1974

10. *Høiby N. & Wiik A.* Antibacterial precipitins and autoantibodies in serum of patients with cystic fibrosis. Scand. J. Resp. Dis. 56 38–46 1975

11. *Høiby N. Andersen V. & Bendixen G.* *Pseudomonas aeruginosa* infection in cystic fibrosis. Humoral and cellular immune response against *Pseudomonas aeruginosa*. Acta path. microbiol. scand. Sect. C, *83* 459 468, 1975.

12. *Johnson K. J. & Ward P. A.* Acute immunologic pulmonary alveolitis. J. Clin. Invest. *54* 349 357 1974

13. *Johnsen S. & Laurell A.-B.* Complement activation in meningococcal septicaemia. Acta path. microbiol. scand. Sect. C, *83* 285 288, 1975

14. *Koch, C. Høiby N. & Wiik A.* Nitroblue Tetrazolium (NBT)-reduction by human peripheral neutrophil granulocytes in the presence of bacterial antigens. Acta path. microbiol. scand. Sect. C, *83* 144–156 1975.

15. *Koffler D. Shur P. H. & Kunkel, H. G.* Immunological studies concerning the nephritis of systemic lupus erythematosus. J. Exp. Med. *126* 607–623 1967

16. *Landry M. & Sams W. M.* Systemic lupus erythematosus. Studies of the antibodies bound to skin. J. Clin. Invest. *52* 1871–1880, 1973

17. *Laurell, C.-B.* Electroimmuno assay. Scand. J. Clin. Lab. Invest. 29 suppl. 124 21–37 1972.

18. *Leber P. D. & McCluskey R. T.:* Immune complex diseases. In *Zweifach B. W., Grant C. & McCluskey R. T.* (ed.) *The Inflammatory Process.* Academic Press, London 1974 *vol. III* second edition, p. 401–441

19. *Mass B., Ikeda, T., Meranze D. R. Weinbaum G. & Kimbel P.* Induction of experimental emphysema. Amer. Rev. Resp. Dis. *106* 384–391 1972.

20. *Mayer, M. M.* Complement and complement fixation. In *Kabat E. A. & Mayer M. M.* *Exp. Immunochemistry* Ed. 2. Springfield, Ill. 1961 p. 133.

21. *McFarlane M. Holzel, A. Brenchly P., Allan J. D., Wallwork J. C. Singer B. E. & Worsley B.* Immune complexes in cystic fibrosis. Brit. Med. J. *1* 423–428, 1975.

22. *McIntosh R. M., Griswold W. R., Chernack W. B. Williams G. Strauss J., Kaufman D. B., Koss M. N., McIntosh, J. R., Cohen, R. & Weil III R.* Cryoglobulins III. Further studies on the nature incidence, clinical, diagnostic, prognostic and immunopathologic significance of cryoproteins in renal disease. Quarterly J. of med. New Series *XLIV:* 285–307 1975.

23. *Nielsen H. & Svehag, S. E.* Detection and differentiation of immune complexes and IgG aggregates by a complement consumption assay. Acta path. microbiol. scand. Sect. C, *84* 261–269 1976

24. *Nielsen H., Olholm P. Daugharty H. & Svehag, S. E.* Detection and kinetics of circulating immune complexes in Herpes zoster sera as revealed by three different assays. Abstract, European Immunology Meeting, Amsterdam 1975

25. *Schiøtz, P. O. & Høiby N.* Precipitating antibodies against *Pseudomonas aeruginosa* in sputum from patients with cystic fibrosis. Specificities and titres determined by means of crossed immunoelectrophoresis with intermediate gel. Acta path. microbiol. scand. Sect. C, *83* 469–475 1975.

26. *Tan E. M. Northway J. D. & Pinnas J. L.* The clinical significance of antinuclear antibodies. Postgrad. Med. *54* 143–150, 1973.

27. *Tan E. M. Schur P. H., Carr R. I. & Kunkel, H. G.* Deoxyribonucleic acid (DNA) and

monstrated 55 per cent (23–83 per cent with 95 per cent confidence) positiveness.

The origin of the soluble immune complexes is unknown. However, localized type III hypersensitivity reactions in the lungs due to complex formation between *P. aeruginosa* antigens and the large number of specific precipitins formed to these antigens have been proposed earlier as a possible mechanism participating in the tissue destruction (6, 9, 25). This hypothesis finds support in experimental studies demonstrating immune complex induced lung damage (4, 12, 19). Dependent on the antigen/antibody ratio, small soluble recirculating immune complexes, or preferably larger complexes will be formed; the latter may remain in the lung tissue, resulting in a type III reaction (18), or they are rapidly cleared from the circulation.

It is known that Ig is found in the dermo-epidermal junction in clinically normal skin from patients with systemic lupus erythematosus and that these patients have circulating immune complexes (1, 16, 29). In the present study, granular deposits of IgM as well as of C1q, C3 and fibrinogen were observed in both groups of patients although they were most frequent in skin biopsies from patients in the CF+P group. The inability to detect deposited *P. aeruginosa* antigens in the skin biopsies from patients in the CF+P group may either be due to blocking by IgM and C-factors or to a restricted multispecificity of the antibody preparation used.

It is noteworthy that *P. aeruginosa* antigens have been detected previously in serum and sputum from CF+P patients (6, 25). Furthermore, one of our CF patients with chronic *P. aeruginosa* infection and strong precipitin response against soluble *P. aeruginosa* antigens as well as two other CF patients with chronic *H. influenzae* infections and a spectrum of precipitins against *H. influenzae* antigens have developed circulating cryoglobulins considered to represent immune complexes (22).

A possible complement consumption *in vivo* by immune complexes was not reflected by changed concentrations of total haemolytic complement or of the complement components under study. Future studies should include investigations of the occurrence of cleavage products of complement factors to determine whether complement activation occurs in CF patients (13).

In accordance with previous results (11) most of the laboratory values reflecting inflammatory reactions and humoral immune responses were not significantly different in the CF+P group and the CF−P group but increased as compared with normal values. The negative RA latex fixation test in all patients is in agreement with previous results which showed that RF of the IgM class is infrequent in CF patients (10).

The occurrence of IgG ON ANF in 24 per cent of the patients also corresponds with earlier findings (10) and is in accordance with the situation in patients with other prolonged respiratory diseases accompanied by tissue destruction (2, 18, 30). Antinuclear antibodies of certain specificities (anti-DNA, anti DNA histone) are known to participate in reactions of the immune complex type *in vivo* (15, 26, 27). The antibody specificities responsible for the ON ANF reactions observed in sera from some of the CF patients should be examined further.

This work was supported by the *Thorvald Madsen Legat*, the *Danish Medical Research Council*, the *National Danish Association against Cystic Fibrosis*, the *Hindsgaul Foundation* and the *National Association against Rheumatic Diseases*.

We thank *Anni Bethien*, *Lis Christiansen* and *George Fam* for skilful technical assistance, the Department of Clinical Chemistry and Dr. *B. Weeke*, Rigshospitalet, Copenhagen, for determination of plasma protein concentrations and Statens Seruminstitut, Copenhagen for total complement determinations.

REFERENCES

1. *Baart de la Faille-Kuyper, E. H.: Lupus erythematosus. An immunohistochemical and clinical study of 485 patients.* M. D. Thesis, Utrecht, 1969.
2. *Bonomo, L., Gillardi, U. & Tursi, A.:* Anti α-globulin and antinuclear factors in chronic

Acta path. microbiol. scand. Sect. C, 85: 65–72, 1977

IN VITRO STIMULATION OF HUMAN LYMPHOCYTES BY *BORDETELLA PERTUSSIS*

V. Andersen, J. B. Hertz, S. F. Sørensen, P. Bækgaard,
P. E. Christensen, W. Ramhøj, G. A. Hansen, A. C. Wardlaw and Y. Sato

Laboratory of Clinical Immunology, Medical Department TA, and
Paediatric Department TG, Rigshospitalet, Copenhagen, Department of Serum and
Vaccines, Statens Seruminstitut, Copenhagen, Department of Microbiology,
University of Glasgow, First Department of Bacteriology, National Institute of Health, Tokyo

Andersen, V., Hertz, J. B., Sørensen, S. F., Bækgaard, P., Christensen, P. E., Ramhøj, W., Hansen, G. A., Wardlaw, A. C. & Sato, Y. *In vitro* stimulation of human lymphocytes by *Bordetella pertussis*. Acta path. microbiol. scand. Sect. C, 85: 65–72, 1977.

Bordetella pertussis (B.p.) induces blast transformation of human lymphocytes; whole killed B.p. are more efficient than extracts obtained by sonication. Similar responses were obtained with each of the four strains used in the Danish pertussis vaccine. B.p. with low amounts of Protective Antigen and Histamine-Sensitizing Factor also induced lymphocyte transformation, but were less toxic to the lymphocytes at high concentrations. The supernatants of B.p. cultures were purified with respect to Lymphocytosis Promoting Factor; evidence is presented that these purified fractions possess T-lymphocyte mitogenic activity. Lymphocytes from all normal humans were stimulated by B.p., including cells from cord blood. Cells from child-bearing women, obtained immediately after delivery, showed a general depression of lymphocyte transformation including the response to B.p. Children with whooping cough had a lower lymphocyte response to B.p. than healthy children. A highly significant correlation was observed between the responses to B.p. and to *E. coli* in the adults and newborn examined. It is concluded that the major part of the lymphocyte transformation induced by B.p. is non-specific.

Key words: *Bordetella pertussis*; lymphocyte transformation; T-lymphocyte mitogen.

Vagn Andersen, Laboratory of clinical immunology, Medical Department TA, Rigshospitalet 7111, Tagensvej 18, DK-2200 Copenhagen N, Denmark.

Received 24.ix.76. Accepted 24.ix.76

In man, the immune response to *Bordetella pertussis* (B.p.) has mainly been studied from the standpoint of protection against disease or in terms of various serological responses (1–6, 13–18). There have been few attempts to analyze either the components of the immune system with which B.p. interacts, or the components of B.p. which stimulate the various parts of the immune system. In this and two companion articles (8–11), the results of studies on lymphocyte and antibody reactivity towards B.p. are reported. The present study concerns the blast transformation response of blood lymphocytes. Optimal conditions for stimulating lymphocytes with B.p.

antibodies to DNA in the serum of patients with systemic lupus erythematosus. J Clin. Invest. 45 1732–1740 1966
Thuesen Pedersen J Lykkegård E. Faro O & Jacobsen L. Lung function in children and young adults with cystic fibrosis. Dan. Med. Bull 21 12–19 1974
Tuffanelli D L. Kay D & Fukuyama, K Dermal-epidermal junction in lupus erythematosus. Arch Derm. 99 652–662 1969
Turner Warwick M & Parkes W R Circulating rheumatoid and antinuclear factors in asbestos workers. Brit. Med. J 3 492–495, 1970
31 *Wiik A & Munthe E.* Restriction among heavy and light chain determinants of granulocyte-specific antinuclear factors. Immunology 23 53–60 1972
32 *Wiik A Jensen E & Friis J* Granulocyte-specific antinuclear factors in synovial fluids and sera from patients with rheumatoid arthritis. Ann. Rheum. Dis. 33 515–522, 1974

Acta path. microbiol. scand. Sect. C 85 65–72 1977

IN VITRO STIMULATION OF HUMAN LYMPHOCYTES BY *BORDETELLA PERTUSSIS*

V. ANDERSEN, J. B. HERTZ, S. F. SØRENSEN, P. BÆKGAARD, P. E. CHRISTENSEN, W. RAMBØJ, G. A. HANSEN, A. C. WARDLAW and Y. SATO

Laboratory of Clinical Immunology, Medical Department TA, and Paediatric Department TG, Rigshospitalet, Copenhagen, Department of Serum and Vaccines, Statens Seruminstitut, Copenhagen, Department of Microbiology, University of Glasgow, First Department of Bacteriology, National Institute of Health, Tokyo

Andersen, V., Hertz, J. B., Sørensen, S. F., Bækgaard, P., Christensen, P. E., Rambøj, W., Hansen, G. A., Wardlaw, A. C. & Sato, Y. *In vitro* stimulation of human lymphocytes by *Bordetella pertussis*. Acta path. microbiol. scand. Sect. C, 85: 65–72, 1977.

Bordetella pertussis (B.p.) induces blast transformation of human lymphocytes; whole killed B.p. are more efficient than extracts obtained by sonication. Similar responses were obtained with each of the four strains used in the Danish pertussis vaccine. B.p. with low amounts of Protective Antigen and Histamine-Sensitizing Factor also induced lymphocyte transformation, but were less toxic to the lymphocytes at high concentrations. The supernatants of B.p. cultures were purified with respect to Lymphocytosis Promoting Factor; evidence is presented that these purified fractions possess T-lymphocyte mitogenic activity. Lymphocytes from all normal humans were stimulated by B.p., including cells from cord blood. Cells from child-bearing women, obtained immediately after delivery, showed a general depression of lymphocyte transformation including the response to B.p. Children with whooping cough had a lower lymphocyte response to B.p. than healthy children. A highly significant correlation was observed between the responses to B.p. and to *E. coli* in the adults and newborn examined. It is concluded that the major part of the lymphocyte transformation induced by B.p. is non-specific.

Key words: *Bordetella pertussis*; lymphocyte transformation; T-lymphocyte mitogen.

Vagn Andersen, Laboratory of clinical immunology, Medical Department TA, Rigshospitalet 7111, Tagensvej 18, DK 2200 Copenhagen N, Denmark.

Received 24.ii.76. Accepted 24.ix.76

In man, the immune response to *Bordetella pertussis* (B.p.) has mainly been studied from the standpoint of protection against disease or in terms of various serological responses (1–6, 15–18). There have been few attempts to analyze either the components of the immune system with which B.p. interacts, or the components of B.p. which stimulate the various parts of the immune system. In this and two companion articles (8, 11), the results of studies on lymphocyte and antibody reactivity towards B.p. are reported. The present study concerns the blast transformation response of blood lymphocytes. Optimal conditions for stimulating lymphocytes with B.p.

are defined The lymphocyte response to various B p strains and to fractions of the supernatant from B p cultures is analyzed. The transformation responses of lymphocytes from normal adults and children, from newborn and their mothers, and from children ill with whooping cough have been studied.

MATERIAL AND METHODS

Culture of B p The four strains constituting pertussis vaccine in Denmark (3803 3825 3843 3860) were cultured separately on a modified Bordet-Gengou medium containing horse blood and charcoal. After 48 hours of culture at 35 C, the bacteria were harvested in 0 9 per cent NaCl. The final concentration of B.p was 1.6 $\times 10^{11}$ per ml (as determined by comparison with the WHO capacity standard)

The effects of different culture conditions were studied in the Canadian vaccine strain 18334 The amounts of Protective Antigen (PA) and Histamine-Sensitizing Factor (HSF) found in B.p cultured in Hornibrook medium is reduced to 1/20-1/40 by culture in high concentrations of either Mg^{++} or nicotinic acid (14 17)

For the study of lymphocyte transforming activity of purified fractions of supernatants from B p. cultures, the Japanese vaccine strain Tohama was employed (16)

Preparations of B p In order to examine the capacity of whole bacteria to stimulate lymphocyte transformation, B p were killed in three different ways a) by heating to 58 for 30 min b) by treatment with 0 01 per cent thiomersal for 5 days, at room temperature or c) by treatment with 0 01 per cent formalin, for 5 days, at room temperature. Methods b) and c) were followed by 4 washings in 0 9 per cent NaCl.

Extracts were prepared from B p by ultrasonic disintegration at a concentration of 1 6 $\times 10^{11}$ B p per ml followed by centrifugation (9) The colloid concentration of the supernatant was 4 0 g/l as measured by refractometry with human IgG as standard.

Fractionation of B p culture supernatant B p strain 3843 was cultured in van Hemert s medium B-2 (7) until after 5 days the B p. concentration was 15 mg dry weight/20 ml The culture was centrifuged the supernatant precipitated w th 4 M ammonium sulphate and the precipitate dissolved to 1/50th of its original volume in phosphate-buffered 1 M NaCl pH 8 0 the protein concentration was 1.5 mg/ml. This is referred to as concentrated culture supernatant.

Supernatant fluid from B.p strain Tohama phase I was concentrated and subjected to starch gel electrophoresis the fractions containing peak Lymphocytosis Promoting Factor (LPF) activity were pooled and concentrated to a protein content of 1.5 mg/ml (16)

Lymphocyte culture The techniques employed for lymphocyte isolation and culture have been described (3) Mononuclear cells were separated from the blood by differential centrifugation on Ficoll-Isopaque. They were washed three times and cultures set up with 10^3 cells per vial, in 500 µl RPMI 1640 with 20 per cent pooled human AB-serum. As mitogens were employed Phytohaemagglutinin (PHA P Difco) at a dilution of 1:600 concanavalin A (ConA, Pharmacia) at a concentration of 20 µg/500 µl and pokeweed mitogen (PWM Gibco) at a dilution of 1 500 these cultures were incubated for 72 h. In addition to B.p., the microbial antigens systems tested were Heat killed (70 C, 30 min) *E. coli*, kindly prepared by Dr *Klars Jensen* and employed at a concentration of 5 $\times 10^4$ per 500 µl an extract of *Candida albicans* kindly donated by Dr *Nils Axelsen* was employed at a protein concentration of 100 µg per 500 µl PPD without chinosol (Statens Seruminstitut) was used at a concentration of 5 µg per 500 µl these cultures were incubated for 120 h. All cultures were set up in triplicate, and the transformation response was measured by the incorporation of ^{14}C-thymidine determined by liquid scintillation counting

Responses are expressed as counts per minute (c.p.m.) A response in antigen-stimulated cultures is considered positive when it is more than 2.5 times the average value of the corresponding unstimulated cultures.

Dose-response and PHA toxicity test For all B p. preparations examined serial four-fold dilutions from 8 $\times 10^8$ bacteria per culture vial were employed. In order to characterize the decrease in lymphocyte stimulation seen at high concentrations of bacteria, PHA was added to replicate cultures after 24 h of culture and the transformation response measured 72 h later If the response to PHA was lower than in corresponding cultures without B.p., this was taken to indicate toxicity of the B p preparation at this concentration.

Immunofluorescence studies on blast cells. The transformed lymphocytes were examined for cell membrane immunoglobulin by fluorescence microscopy after incubation with FITC-conjugated polyvalent antihuman immunoglobulin (Dakopatts, Copenhagen) As positive controls, PWM stimulated lymphocyte cultures were examined in parallel, since a proportion of the lymphoblasts in these cultures stain for membrane immunoglobulin

Lymphocyte donors. Healthy adults were between 25 and 45 years old and included persons with a history of whooping cough in childhood, persons who had received pertussis vaccination, and persons who had neither

Cord blood samples were studied, together with

TABLE 1 *Transformation of Lymphocytes from an Adult by Various Preparations of B.p*

	Maximal c.p.m.	Optimal concentration (per culture of 300 µl)
Heat-killed B.p.	4895	5×10^7 B.p.
Thiomersal-killed B.p.	4509	5×10^7 B.p.
Formalin-killed B.p.	4896	5×10^6 B.p.
Extract of sonicated B.p.	2820	12 µg protein
B.p. culture supernatant	901	undiluted
Concentrated supernatant	1627	6 µg protein
Unstimulated	606	

With all preparations, dose titration was carried out with the exception of undiluted culture medium, higher concentrations gave lower c.p.m. and demonstrable toxicity (PHA toxicity test)

blood samples from the mothers obtained within 15 min of delivery. In all cases the period of gestation had been of normal length and without complications, and the delivery was likewise uncomplicated.

Healthy children, fully vaccinated against pertussis and without a history of whooping cough, were sampled at age 11–20 months.

Children with whooping cough proven by culture of B.p. were studied once at least three weeks after the onset of symptoms (we thank Prof. *V. Faber* and the nursing staff of Blegdamshospitalet for their kind help in obtaining the blood samples).

Statistical methods. For evaluation of lymphocyte transformation responses to different B.p. preparations or between *different groups of persons*, the rank sum test was employed. The correlation between the increases in thymidine incorporation induced by different microorganisms was calculated *by the method of least squares.*

RESULTS

All 19 adults studied showed a lymphocyte transformation response to B.p. The results of *a typical experiment are given in Table 1.* Whole bacteria consistently induced higher lymphocyte responses than extracts obtained by sonication. Bacteria killed *by heat*, thiomersal, and formalin worked equally well, provided the chemically treated B.p. were washed extensively before being added to the lymphocyte cultures. PHA toxicity studies likewise failed to indicate higher toxicity of these preparations as compared to the heat killed bacteria.

A typical dose-response curve using heat killed B.p., is given in Fig. 1. With lymphocytes from different donors, the concentration that induces maximal *transformation* was in the range 0.5–80 $\times 10^7$ B.p. per culture of 10^5 mononuclear cells. The most frequent optimum was 5×10^7 *B.p. per culture.* Toxicity studies showed that if 5×10^7 B.p./culture was the optimal concentration, 20×10^7 B.p./*culture* gave *slight inhibition of the* response to PHA, and 80×10^7 B.p./culture completely abolished the PHA response.

The results obtained with each of the four Danish vaccine strains separately showed no significant differences (Table 2). B.p. grown *under* conditions resulting in very low

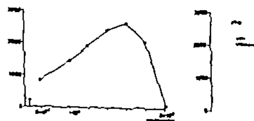


Fig. 1. Transformation of lymphocytes from an adult induced by varying concentrations of B.p. Abscissa: number of heat-killed B.p. added per culture of 10^5 mononuclear cells. The c.p.m. in cultures stimulated by PPD (×), *E. coli* (●) or extract of *Candida albicans* (+) are given for comparison. The small bar indicates c.p.m. of unstimulated cultures.

are defined. The lymphocyte response to various B.p. strains and to fractions of the supernatant from B.p. cultures is analyzed. The transformation responses of lymphocytes from normal adults and children, from newborn and their mothers, and from children ill with whooping cough have been studied.

MATERIAL AND METHODS

Culture of B.p. The four strains constituting pertussis vaccine in Denmark (3803, 3825, 3843, 3860) were cultured separately on a modified Bordet-Gengou medium containing horse blood and charcoal. After 48 hours of culture at 35 C, the bacteria were harvested in 0.9 per cent NaCl. The final concentration of B.p. was 1.6 $\times 10^{11}$ per ml (as determined by comparison with the WHO capacity standard).

The effects of different culture conditions were studied in the Canadian vaccine strain 18334. The amounts of Protective Antigen (PA) and Histamine-Sensitizing Factor (HSF) found in B.p. cultured in Hornibrook medium is reduced to 1/20-1/40 by culture in high concentrations of either Mg^{++} or nicotinic acid (14, 17).

For the study of lymphocyte transforming activity of purified fractions of supernatants from B.p. cultures, the Japanese vaccine strain Tohama was employed (16).

Preparations of B.p. In order to examine the capacity of whole bacteria to stimulate lymphocyte transformation, B.p. were killed in three different ways: a) by heating to 58 for 30 min, b) by treatment with 0.01 per cent thiomersal, for 5 days, at room temperature, or c) by treatment with 0.01 per cent formalin, for 5 days, at room temperature. Methods b) and c) were followed by 4 washings in 0.9 per cent NaCl.

Extracts were prepared from B.p. by ultrasonic disintegration at a concentration of 1.6 $\times 10^{11}$ B.p. per ml followed by centrifugation (9). The colloid concentration of the supernatant was 4.0 g/l as measured by refractometry with human IgG as standard.

Fractionation of B.p. culture supernatant. B.p. strain 3843 was cultured in van Hemert's medium B-2 (7) until after 5 days the B.p. concentration was 15 mg dry weight/20 ml. The culture was centrifuged, the supernatant precipitated with 4 M ammonium sulphate and the precipitate dissolved to 1/50th of its original volume in phosphate buffered 1 M NaCl, pH 8.0; the protein concentration was 1.3 mg/ml. This is referred to as concentrated culture supernatant.

Supernatant fluid from B.p. strain Tohama phase I was concentrated and subjected to starch gel electrophoresis: the fractions containing peak Lymphocytosis Promoting Factor (LPF) activity were pooled and concentrated to a protein content of 1.5 mg/ml (16).

Lymphocyte culture. The techniques employed for lymphocyte isolation and culture have been described (9). Mononuclear cells were separated from the blood by differential centrifugation on Ficoll-Isopaque. They were washed three times and cultures set up with 10^5 cells per vial, in 500 µl RPMI 1640 with 20 per cent pooled human AB-serum. As mitogens were employed Phytohaemagglutinin (PHA P, Difco) at a dilution of 1:600, concanavalin A (ConA, Pharmacia) at a concentration of 20 µg/500 µl and pokeweed mitogen (PWM, Gibco) at a dilution of 1:500; these cultures were incubated for 72 h. In addition to B.p. the microbial antigens systems tested were: Heat killed (70 C, 30 min) *E. coli*, kindly prepared by Dr. *Klaus Jensen* and employed at a concentration of 5 $\times 10^4$ per 500 µl; an extract of *Candida albicans* kindly donated by Dr. *Nils Axelsen* was employed at a protein concentration of 100 µg per 500 µl; PPD without chinosol (Statens Seruminstitut) was used at a concentration of 5 µg per 500 µl; these cultures were incubated for 120 h. All cultures were set up in triplicate and the transformation response was measured by the incorporation of ^{14}C-thymidine, determined by liquid scintillation counting.

Responses are expressed as counts per minute (c.p.m.). A response in antigen-stimulated cultures is considered positive when it is more than 2.5 times the average value of the corresponding unstimulated cultures.

Dose-response and PHA toxicity test. For all B.p. preparations examined, serial four-fold dilutions from 8 $\times 10^8$ bacteria per culture vial were employed. In order to characterize the decrease in lymphocyte stimulation seen at high concentrations of bacteria, PHA was added to replicate cultures after 24 h of culture, and the transformation response measured 72 h later. If the response to PHA was lower than in corresponding cultures without B.p. this was taken to indicate toxicity of the B.p. preparation at this concentration.

Immunofluorescence studies on blast cells. The transformed lymphocytes were examined for cell membrane immunoglobulin by fluorescence microscopy after incubation with FITC-conjugated polyvalent antihuman-immunoglobulin (Dakopatts, Copenhagen). As positive controls PWM stimulated lymphocyte cultures were examined in parallel, since a proportion of the lymphoblasts in these cultures stain for membrane immunoglobulin.

Lymphocyte donors. Healthy adults were between 25 and 45 years old and included persons with a history of whooping cough in childhood, persons who had received pertussis vaccination, and persons who had neither.

Cord blood samples were studied, together with

TABLE 3 *Lymphocyte Transformation Induced by LPF-enriched Fractions of Supernatant from B.p Cultures. Averages of Three Experiments*

	Maximal c.p.m. ± S.E.M.	Optimal concentration (per culture of 500 μl)
Fraction Z-F	30191 ± 2276	120 or 50 μg protein
Second peak	34027 ± 826	3 or 0.8 μg protein
Heat-killed B p.	4,03 ± 1553	2×10^8 or 5×10^9
Unstimulated	334 ± 138	

TABLE 4 *Transformation of Lymphocytes from Normal Adults Induced by B.p and by Other Microbial Preparations*

	No. positive/ No. tested	Average of maximal response c.p.m. ± S.E.M	Correlation to B p. response r
B p.	18/18	2477 ± 284	
E. coli	18/18	2097 ± 205	0.71 ($p<0.01$)
C albicans-extract	18/18	2260 ± 211	0.64 ($p<0.02$)
PPD	18/18	3764 ± 617	0.69 ($p<0.01$)
Unstimulated		376 ± 85	

the transformed cells showed membrane immunofluorescence.

As mentioned above the lymphocytes of all normal adults studied showed transformation when exposed to heat killed B p. The response was not higher in those with a history of whooping cough or pertussis vaccination than in those without. There was a significant positive correlation, in the individual persons tested, between the responses to B p. and those to other microorganisms (Table 4).

The lymphocytes of all newborn studied showed a transformation response to B.p. (Table 5). As is well known, the unstimulated thymidine incorporation of cord blood lymphocytes is much higher than in adults (12. Even so, the increased incorporation induced by B p. is significant, both in absolute values (Table 5) and expressed as the ratio B p.-stimulated : unstimulated cells. *E. coli* and in most cases *C albicans* likewise induced significant transformation, but not PPD. the individual response to B p. and to *E. coli* showed a significant correlation ($r = 0.76$, $p<0.05$). The mothers showed low mitogen responses and low or no responses to B.p. and the other antigens tested.

Children who recently had finished their pertussis vaccination course showed B.p.-induced transformation values which were not significantly different from those of adults (Table 6).

Eight children, hospitalized for pertussis were examined 3–6 weeks after the onset of symptoms. B p.-induced thymidine incorporation was observed in cultures of mononuclear cells obtained from these patients, but the maximal response was lower than in healthy children (Table 6). the optimal B.p. concentration was not different.

amounts of PA and HSF did not induce less lymphocyte transformation than the same strain grown under normal conditions their toxicity at high concentrations was less than that of B p with normal amounts of PA and HSF (Fig 2)

Neither fresh medium nor unconcentrated culture supernatant from 5 day-old B p cultures stimulated lymphocyte transformation.

TABLE 2 *Transformation of Lymphocytes from Adults by Heat-killed B p of the Danish Vaccine Strains Singly and in Combination Averages of 3 Experiments*

	Maximal c.p.m. ± S E.M
Danish vaccine strain 3803	2813 ± 108
Danish vaccine strain 3825	2450 ± 368
Danish vaccine strain 3843	3472 ± 707
Danish vaccine strain 3860	2806 ± 586
All four strains	2711 ± 477
Unstimulated	225 ± 117

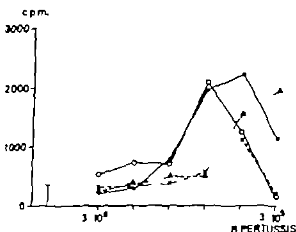


Fig 2 Transformation of lymphocytes from an adult by the Canadian vaccine strain 18334

○ —○ grown under ordinary conditions.
● —● grown at high concentration of Mg^{2+} (suppresses production of PA and HSF)
× --× grown at high concentration of nicotinamide (normal levels of PA and HSF)
Δ- -Δ grown at high concentration of nicotinic acid (suppresses production of PA and HSF)

Abscissa number of heat killed B.p added per culture of 10^5 mononuclear cells. *The small bar indicates* c.p.m. of unstimulated cultures.

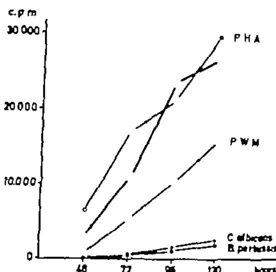


Fig 3 Transformation of lymphocytes from an adult induced by fraction Z-F from B.p. culture supernatant (heavy line) For comparison, the responses to phytohaemagglutinin (PHA) pokeweed mitogen (PWM) *C albicans*-extract, and heat-killed B p are given.

But supernatant concentrated by precipitation with 4 M ammonium sulphate induced *slight lymphocyte transformation* (Table 1)

When the supernatant of B p culture is fractionated by starch gel electrophoresis, a fraction called Z-F by *Sato & Arai* (16) contains the highest LPF activity This fraction induced a tremendous degree of lymphocyte transformation the thymidine incorporation rose much faster than that induced by heat killed B p and was similar to that induced by PHA (Fig 3) Further purification of LPF by sucrose density gradient centrifugation and agarose column chromatography (16) gave a preparation ("Second peak") with similar lymphocyte transformation activity at much lower concentration (Table 3)

The lymphoblasts of cultures stimulated by fraction Z F were examined for cell membrane immunoglobulin by means of immunofluorescence, using a fluorescein labelled polyvalent antiglobulin antiserum Only 0.5 per cent of the lymphoblasts showed fluorescence which means that practically all transformed lymphocytes were without detectable membrane-bound immunoglobulin. As controls, PWM induced lymphoblasts were examined in parallel in these cultures, 50 per cent of

the lack of demonstrable immunoglobulin on the surface of the transformed cells. T cell blasts have been shown to be devoid of surface immunoglobulin while B cell blasts possess demonstrable cell membrane immunoglobulin (5 12). Mitogenicity of a B.p. preparation for murine T lymphocytes has been described by *Morse & Kong* (15).

All healthy persons showed a lymphocyte transformation response to B.p. the magnitude of the response was correlated to the stimulation induced by other microbial preparations, in particular by *E. coli*. Mononuclear cells obtained from childbearing women immediately after delivery showed a general depression of lymphocyte transformation, with low responses to mitogens and low or absent responses to antigens including B.p. This indicates that parturition can be included among the non-specific stress factors depressing lymphocyte transformation. The lymphocytes of the newborn all responded to B.p. and to *E. coli* as well, again with a highly significant correlation between the two responses.

Children ill with pertussis showed a lower response to B.p. than healthy children: this was mirrored by lower responses also to *E. coli* and *C. albicans*-extract. Decreased lymphocyte responses to several microbial antigens have been described in patients with infectious diseases (10 4) and may thus reflect a generalized hyporesponsive state due to the illness.

In conclusion the major part of the lymphocyte transformation response induced by B.p. seems to be non-specific. There was a highly significant correlation between the responses to B.p. and to *E. coli* in all the donor groups examined. This may indicate that the serologically demonstrable cross-reactivity between components of B.p. and of other microorganisms (11) is a determining factor also for lymphocyte stimulation. The demonstration of a mitogenic factor in purified fractions of B.p. culture medium may also be of relevance for this apparent non-specificity but whether the mitogenic factor is decisive for the lymphocyte transformation induced by whole heat-killed B.p. remains to be established.

We thank *Paul Gleating*, M.D., for his inspiring questions which started our work. We are indebted to *Vit Walbull* and *Jett Harding* for competent technical assistance, and to *Kirsten Garmer* for excellent secretarial help. The work was supported by the *Danish Medical Research Council* and by the *Dasll Foundation*.

REFERENCES

1. *Aftandelians R. & Connor J. D.*: Bactericidal antibody in serum during infection with *Bordetella pertussis*. J. Infect. Dis. *128* 555–558, 1973.
2. *Andersen V. & Andersen E.*: Changes in blood lymphocytes during the neonatal period. Acta Paed. Scand. *63* 266–270 1974.
3. *Andersen, V., Thomsen M. & Cohn J.*: Immune deficiency in sex-linked hereditary thrombocytopenia. Acta path. microbiol. scand. Sect. C, *83* 177–183 1975.
4. *Andersen V., Hansen N. E., Karle H., Lind I., Høiby N. & Weeke B.*: Sequential studies of lymphocyte responsiveness and antibody formation in acute bacterial meningitis. (In press) Clin. Exp. Immunol.
5. *Chess L., MacDermott R. P. & Schlossman S. F.*: Immunologic functions of isolated human lymphocyte subpopulations. I. Quantitative isolation of human T and B cells and response to mitogens. J. Immunol. *113* 1113–1121 1974.
6. *Dalby J. M. & Stephens, S.*: Pertussis antibodies in the sera of children exposed to *Bordetella pertussis* by vaccination or infection. J. Hyg., Camb. *71* 193–207 1973.
7. *van Hemert P.*: Vaccine production as a unit process. Thesis, Delft 1971.
8. *Hertz, J. B., Høiby N., Andersen, V. & Bækgaard P.*: Crossed immunoelectrophoretic analysis of *Bordetella pertussis* antigens and of corresponding antibodies in human sera. Acta path. microbiol. scand. Sect. B, *84* 386–394 1976.
9. *Høiby N.*: Cross-reactions between *Pseudomonas aeruginosa* and thirty-six other bacterial species. Scand. J. Immunol. *4* suppl. 2: 187 196, 1975.
10. *Høiby N., Andersen V. & Bendixen G.*: *Pseudomonas aeruginosa* infection in cystic fibrosis. Humoral and cellular immune responses against *Pseudomonas aeruginosa*. Acta path. microbiol. scand. Sect. C, 83 459–468, 1975.
11. *Høiby N. Hertz, J. B. & Andersen V.*: Cross-

TABLE 5 *Lymphocyte Transformation responses to B p of Lymphocytes from 7 Newborn Infants and from their mothers Average Values ± S.E.M*

	Newborn c.p.m. ± S.E.M.	Mothers c.p.m. ± S E.M.
72 hours' culture		
PHA	19010 ± 3758	9606 ± 1616
ConA	12450 ± 4069	?468 ± 764
PWM	6407 ± 1692	2014 ± 413
Unstimulated	4?1 ± 70	198 ± 45
120 hours' culture		
B p	3158 ± 940	341 ± 140
E coli	3086 ± 674	332 ± 105
C albicans	1488 ± 242	359 ± 128
PPD	748 ± 35	372 ± 132
Unstimulated	651 ± 202	74 ± 26

TABLE 6 *Lymphocyte Transformation Responses in 4 Healthy Children Fully Vaccinated against Pertussis and in 8 Children with Whooping Cough*

	Unstimulated c.p.m. ± S.E.M	Maximum response to B p c.p.m. ± S.E.M	Optimum concentration (per culture of 500 μl)
4 healthy children (*age 11–20 months*)	266 ± 119	3143 ± 859	8×10^{6}–8×10^{7} B.p.
8 children with pertussis (age 2–?7 months)	115 ± 27	1763 ± 651	8×10^{6}–3×10^{7} B.p.

DISCUSSION

Human lymphocytes are activated to blast transformation by B p *in vitro* Whole killed B p induce a higher degree of lymphocyte transformation than does an extract of sonicated bacteria. B.p killed by heat and by chemical treatment are equally effective provided the latter are thoroughly washed. We found no differences in transformation inducing ability between the four strains which compose the Danish pertussis vaccine. If subjected during culture to factors which decreased the content of PA and HSF the B.p. were equally able to induce lymphocyte transformation but were less toxic at high concentrations (Fig 2)

The optimal concentration averaged 5 × 10 heat killed B p per 500 μl culture containing 10^{6} mononuclear cells it was practically constant in the same person over a period of 9 months, but varied by a factor of 250 between different individuals. Thus, although there is a fairly broad plateau with respect to antigen concentrations giving a high response, it is necessary to use a range of concentrations in order to obtain with certainty the maximum response of an individual not previously tested.

The lymphocyte-activating capacity (Fig 3) of fractions of B p culture supernatant, purified with respect to LPF activity is probably due to a non-specific mitogenic effect. The magnitude and the time sequence of the response is comparable to that induced by PHA. This indication that the preparation is mitogenic for T lymphocytes is confirmed by

Acta path. microbiol. scand. Sect. C, 85: 73–77, 1977

ISOTOPE LABELLED α-AMINO-ISOBUTYRIC ACID AS AN INDICATOR IN CYTOTOXICITY TESTS

TORSTEIN LYBERG

Department of Microbiology, Dental Faculty, University of Oslo, Blindern, Oslo, Norway

Lyberg, T. Isotope-labelled α-amino-isobutyric acid as an indicator in cytotoxicity tests. Acta path. microbiol. scand. Sect. C, 85: 73–77, 1977.

The effect of rabbit antisera on the uptake and release of α-amino-isobutyric acid (AIBA) in L5- and P 388 cells was investigated. In the presence of homologous antiserum and complement, the uptake of AIBA was inhibited. In the absence of complement, no effect of antiserum was seen. Using prelabelled cells, the efflux of AIBA was greatly accelerated in the presence of antiserum and complement. The AIBA-uptake method was compared with the trypan blue exclusion test and the ^{51}Cr release technique. The AIBA-uptake method was more sensitive in quantitative cytotoxic studies.

Key words: Cytotoxicity tests; α-amino-isobutyric acid; isotope labelled; indicator.

T. Lyberg, Department of Microbiology, Dental Faculty, University of Oslo, Blindern, Oslo 3, Norway.

Received 1.i.76. Accepted 1.x.76

When animal cells are treated *in vitro* with serum containing specific antibodies against cell surface antigens, distinct morphological alterations in the cell membrane complex occur including extensive projection and folding (5). Introduction of complement into the *in vivo* system amplifies the morphological changes towards cytoplasmic swelling, vesiculation and loss of cytoplasmic basophilia (5).

In order to acquire a better understanding of the concomitant biochemical consequences of such treatment, we have investigated the effect of antiserum on transmembrane transport of α-amino-isobutyric acid (AIBA) in animal cells. The uptake of this amino acid has earlier been used as an indicator of cell viability (2). AIBA exhibits the transport characteristics of common amino acids. It is, however, not metabolized intracellularly and can thus be recovered in its original form. *Dickson* (2) concluded that the maintenance of an AIBA concentration gradient is a reliable index of cell viability.

A suitable method by which to measure the actual uptake of metabolites has been shown to include the use of radioactive labelled inulin in the sample as inulin is physiologically inert and completely excluded from cells (4).

In the present paper, the uptake of isotope-labelled α-amino-isobutyric acid [^{14}C-AIBA] has been used to study cell damage caused by antibody directed against cell surface antigens. The results have been compared with those obtained by the widely used trypan blue cytotoxicity test described by *Gorer & O'Gorman* (6) and to the ^{51}Cr release method (9).

reactions between *Bordetella pertussis* and twenty-eight other bacterial species. In press) Acta path. microbiol scand Sect. B *84* 395–400 1976

12 *Jondal M* Surface markers on human B and T lymphocytes. IV Distribution of surface markers on resting and blast transformed lymphocytes. Scand J Immunol. *3* 739–755 1974

13 *Morse S I & Kong A S* Generation of cytotoxic lymphocytes by the lymphocytosis promoting factor (LPF) of *Bordetella pertussis* 1975 European Immunology Meeting Amsterdam

14 *Parton R. & Wardlaw A C* Cell envelope proteins of *Bordetella pertussis* J med. microbiol *8* 47–57 1975

15 *Pittman M B. pertussis*—bacterial and host factors in the pathogenesis and prevention of whooping cough. In *Mudd S* (Ed.) Infectious Agents and Host Reactions, Saunders, Philadelphia, London and Toronto 1970, pp 239–70

16 *Sato Y & Arai H* Leukocytosis-promoting factor of *Bordetella pertussis* purification and characterization. Infect. Immun. *6* 889-904 1972

17 *Wardlaw A D Parton R & Hooker M J* Loss of protective antigen, histamine sensitizing factor and envelope polypeptides in cultural variants of *Bordetella pertussis*. J med. microbiol. *9* 89–100 1976.

18. World Health Organization Immunization against whooping cough WHO Chronicle *29* 365–367 1975

Acta path. microbiol. scand. Sect. C, 85 73–77 1977

ISOTOPE LABELLED α-AMINO ISOBUTYRIC ACID AS AN INDICATOR IN CYTOTOXICITY TESTS

TORSTEIN LYBERG

Department of Microbiology Dental Faculty University of Oslo Blindern, Oslo, Norway

Lyberg, T. Isotope-labelled α-amino-isobutyric acid as an indicator in cytotoxicity tests. Acta path. microbiol. scand. Sect. C, 85 73 77 1977

The effect of rabbit antisera on the uptake and release of α-amino-isobutyric acid (AIBA) in LS- and P 388 cells was investigated. In the presence of homologous a tisera and complement, the uptake of AIBA was inhibited. In the absence of complement, no effect of antiserum was seen. Using prelabelled cells, the efflux of AIBA was greatly accelerated in the presence of antiserum and complement. The AIBA-uptake method was compared with the trypan blue exclusion test and the Cr release technique The AIBA-uptake method was more smooth in quantitati cytotoxic studies.

Key words Cytotoxicity tests α-amino-isobutyric acid isotope labelled indicator

T Lyberg, Department of Microbiology Dental Faculty University of Oslo, Bl ndern, Oslo 3 Norway

Received 1 1 76 Accepted 1.x.76

When animal cells are treated *in vitro* with serum containing specific antibodies against cell surface antigens, distinct morphological alterations in the cell membrane complex occur including extensive projection and folding (5) Introduction of complement into the *in vitro* system amplifies the morphological changes towards cytoplasmic swelling vesiculation and loss of cytoplasmic basophilia (5)

In order to acquire a better understanding of the concomitant biochemical consequences of such treatment, we have investigated the effect of antiserum on transmembrane transport of α-amino-isobutyric acid (AIBA) in animal cells. The uptake of this amino acid has earlier been used as an indicator of cell viability (2) AIBA exhibits the transport characteristics of common amino acids. It is, however not metabolized intracellularly and can thus be recovered in its original form. *Dickson* (2) concluded that the maintenance of an AIBA concentration gradient is a reli able index of cell viability

A suitable method by which to measure the actual uptake of metabolites has been shown to include the use of radioactive la belled inulin in the sample as inulin is physiologically inert and completely excluded from cells (4)

In the present paper the uptake of isotope labelled α-amino-isobutyric acid [^{14}C-AIBA] has been used to study cell damage caused by antibody directed against cell surface anti gens. The results have been compared with those obtained by the widely used trypan blue cytotoxicity test described by *Gorer & O Gorman* (6) and to the ^{51}Cr release method (9)

reactions between *Bordetella pertussis* and twenty-eight other bacterial species. In press) Acta path. microbiol scand. Sect. B *84* 395–400 1976

12 *Jondal M* Surface markers on human B and T lymphocytes. IV Distribution of surface markers on resting and blast-transformed lymphocytes. Scand. J Immunol *3* 739–755 1974

13 *Morse S I & Kong A S* Generation of cytotoxic lymphocytes by the lymphocytosis promoting factor (LPF) of *Bordetella pertussis* 1975 European Immunology Meeting Amsterdam.

14 *Parton R & Wardlaw A C.* Cell envelope proteins of *Bordetella pertussis* J med. microbiol. *8* 47–57 1975

15 *Pittman M B. pertussis*—bacterial and host factors in the pathogenesis and prevention of whooping cough In *Mudd S* (Ed.) Infectious Agents and Host Reactions, Saunders, Philadelphia, London and Toronto 1970 pp. 239–70

16 *Sato Y & Arai H* Leukocytosis-promoting factor of *Bordetella pertussis* purification and characterization. Infect. Immun. *6* 889–904 1972

17 *Wardlaw A D Parton R. & Hooker M J* Loss of protective antigen histamine-sensitizing factor and envelope polypeptides in cultural variants of *Bordetella pertussis* J med. microbiol. *9* 89–100 1976

18. World Health Organization Immunization against whooping cough. WHO Chronicle 29 365–367 1975

of the water-extract were added directly to 15 ml of scintillation liquid.

^{14}C and ^{3}H were counted in Packard Tri-Carb liquid scintillation spectrometer (Model 3003) operated at 0° C. Disintegrations were calculated according to the simultaneous equation method (8). The amount of extracellular AIBA in the cell pellet was calculated as follow

$$^{14}\text{C-AIBA ex. cell.} = {}^{3}\text{H inulin ex. cell.} \frac{^{14}\text{C-AIBA medium}}{^{3}\text{H-inulin medium}}$$

To calculate intracellular AIBA, the obtained value for extracellular AIBA was subtracted from the total ^{14}C count of the pellet.

RESULTS

Comparison of Techniques by Dose Response Curves

The uptake of ^{14}C-AIBA was determined in LS-cells which had been treated with a fixed amount of complement and varying concentrations of rabbit anti-LS serum. The results were compared with those obtained by the trypan blue cytotoxicity test.

The AIBA assay detected cytotoxic damage at concentrations of antiserum lower than those to be detected by the trypan blue cytotoxicity test (Fig. 1). The figure shows that the sensitivity increased by a factor of about two using the AIBA-uptake method. The results of 15 experiments showed a mean value of 2.0 (range 1.7–2.3). Similar

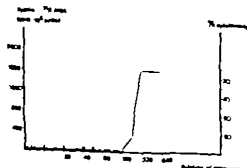


Fig. 1. Effect of varying quantities of rabbit antiserum and complement (GPS 1:4) on LS-cells. Uptake of ^{14}C-AIBA and trypan blue exclusion tests. Δ——Δ trypan blue ○——○ ^{14}C-AIBA uptake.

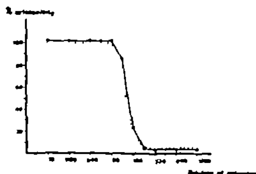


Fig. 2. Dose response curves for rabbit anti-LS serum on LS-cells in the presence of constant amount of complement. ^{51}Cr release and trypan blue exclusion tests. Δ– – –Δ trypan blue ●——● ^{51}Cr release

results were obtained if P-388 cells and the corresponding antiserum were used.

In assays comparing ^{51}Cr release and trypan blue exclusion as criteria of cell death, no difference in cytotoxic titre was found (Fig. 2).

Various polysaccharides and lipopolysaccharides, including inulin, activate the alternate pathway of the complement system. A study was undertaken to show whether inulin in the concentration at current interest would activate the late-acting complement components.

A cytotoxic assay was set up using the trypan blue exclusion method. The test was performed in triple series, one containing antiserum and complement, the other including also inulin in concentration 0.2 μCi/ml medium, and the third including complement and inulin without antiserum. Inulin did not activate the complement components in the fluid phase and had no influence on the antibody dose response curve.

Kinetics of Complement Lysis of Antibody-Treated Cells

A suspension of LS cells was sensitized for 30 min with a subagglutinating though cytotoxic dose of antiserum. GPS was then added to the sensitized cells. It was allowed to act for various intervals of time after which the

MATERIALS AND METHODS

Cell lines Suspension cultures of LS-cells (mouse fibroblasts) and P 388 D cells (a strain of mouse lymphoblastoma cell) were grown in Eagle's minimum essential medium for suspension culture (Grand Island Biological Company USA) Methocel 1 2 g/l peptone 50 g/l, pluronic acid 1 g/l penicillin 100,000 IU/l, streptomycin 100 mg/l and 10 per cent inactivated (56 C 30 min) bovine serum were added Before use, the cells were spun down in a centrifuge at 800 × g for 5 min washed and resuspended to the desired concentration in Hanks Balanced Salt Solution (HBSS) pH 7 4

Antisera Antisera were prepared in rabbits by injecting cells which had been washed three times in HBSS and finally resuspended in the same solution. With a view to production of LS-antisera, rabbits were injected intravenously twice weekly for 2 months, each time using 1 ml cell suspension containing 10^7 cells. A similar procedure was used for obtaining anti-P 388 sera except that the immunization started with two subcutaneous injections.

The animals were bled one week after the last injection Blood was allowed to clot at room temperature for 1 h It was stored overnight at 5 C, and serum was separated by centrifugation at 800 × g for 5 min in the cold. Sera were inactivated at 56 C for 30 min and stored at −20 C

The antisera showed cross-reactivity between LS- and P 388 cells. After absorption with the heterologous cells they retained specific cytotoxic antibodies Inactivated sera from the pre-immunised animals showed no cytotoxicity against either cell type.

Complement Different sources of complement were used in the cytotoxicity assays. Blood was obtained from healthy human subjects as well as from members of rabbit and guinea-pig species serum was separated, stored at −20 C and thawed immediately before use. Guinea-pig serum (GPS) showed no inherent cytotoxicity to LS-cells and could be used without preceding absorption. Serum from man and rabbit contained unspecific cytotoxic antibodies which had to be absorbed before use. For this reason and because of the higher efficacy of guinea-pig complement in the actual system, GPS was chosen as source of complement in the following experiments.

Trypan blue cytotoxicity test The following mixture was incubated at 37 C for 45 min

0 1 ml cell suspension (3 × 10^6 cells/ml)
0 1 ml antiserum
0 1 ml GPS (1 4)

Reagents were added in the order given. After incubation, 0 1 ml 0 75 per cent trypan blue was added to the reaction mixture and incubation continued for 45 min. HBSS pH 7 4 was used as diluent.

Cells were counted in a Bürker haemocytometer and the cytotoxic titre was defined as the highest dilution of antiserum giving more than 50 per cent dead cells.

^{51}Cr assays Chromium-51 release assay was performed by a modification of the method described by *Wigzell* (9) 20 ml suspensions of LS-cells in HBSS (10^6 cells/ml) were incubated with 80 µCi ^{51}Cr (Na_2CrO_4 Institutt for Atomenergi, Isotope Department Kjeller Norway spec. act. 1·6 µCi/µg) for 1 h at 37 C. After incubation, the cells were washed three times in HBSS, they were adjusted to 3 × 10^6/ml and used in the cytotoxicity test.

Dilutions of antiserum and complement GPS (1·4) were added in volumes of 0.5 ml, respectively to 0.5 ml of labelled cells and the mixture was incubated at 37 C for 90 min. The cells were then sedimented by centrifugation and 1 ml of the supernatant was removed to be counted in a Packard gamma spectrometer (Packard Instrument Co, Downers Grove Ill., USA)

Controls composed of cells alone and of cells and complement were included. The maximum release value was arbitrarily considered as the amount of ^{51}Cr to be released in the first test tubes in the dilution series. The percentage of lysis was determined by the following formula

$$\frac{\text{cpm of experiment—cpm of control}}{\text{cpm of max. release—cpm of control}} \quad 100$$

AIBA uptake assays The effect of antiserum and complement was studied in a mixture containing 0.5 ml of cell suspension (3 × 10^6 cells/ml) 0.5 ml of antiserum dilution and 0.5 ml of GPS (1 4) added in the order given. HBSS was used as diluent.

After incubation of the reaction mixture for 45 min, the cells were pulse-labelled for 45 min by addition of ^{14}C-AIBA (spec. act. 58 mCi/mmol) and ^{3}H inulin (spec. act 300 mCi/mmol) to a final concentration of 0 02 µCi/ml and 0.2 µCi/ml, respectively The radiochemicals were obtained from The Radiochemical Centre, Amersham, U.K.

In order to estimate the amount of AIBA and inulin in the medium, the cells were centrifuged at 800 × g for 5 min and 50 µl of the supernatant fluid were transferred to counting vials containing 15 ml scintillation liquid (PPO 4 gm, dimethyl-POPOP 0 05 gm Triton X 100 (scintillation grade) 333 ml and toluene 667 ml)

In order to estimate the extracellular and membrane-associated AIBA and inulin in the cell pellet, the remaining supernatant fluid was carefully removed, 1 ml of water was added to the cell pellet and the suspension was boiled for 5 min. 50 µl

e.g. the anticomplementary effect of trypan blue, the concentration of the dye and the time of incubation with the dye (3). Also the counting error and the subjective discrimination between live and dead cells were avoided. The method also increased the sensitivity with a view to detection of cytotoxic antibodies.

The release of ^{51}Cr has gained widespread use as a suitable technique for quantitative cytotoxic studies. Our experiments including comparison of ^{51}Cr release and trypan blue exclusion assays, indicated a close correlation between antiserum dose response curves. This is in agreement with observations by *Wigzell* (9) and *Detrick-Hooks et al.* (1).

The uptake of ^{14}C-AIBA seems to be a method more sensitive than both the ^{51}Cr release and trypan blue exclusion assays for the quantitation of cytotoxic antibodies. Besides, the method is accurate, simple to perform and easy to interpret. The greater sensitivity is hardly due to activation of the alternate pathway of complement caused by inulin, since the inulin concentration is considered far too low to initiate the complement sequence. The concentration of inulin used by *Götze & Müller-Eberhard* (7) in their study of the C3 activator system was 1,000–200,000 times that used in the present experiments.

The AIBA-uptake method is somewhat more time-consuming than the trypan blue test and it must be held on a macro scale to meet the statistical requirements for liquid scintillation counting. The AIBA uptake method is therefore considered useful for special purposes, not for screening tests and routine use where the dye exclusion tests are more convenient.

REFERENCES

1. *Detrick-Hooks B., Borsos T. & Rapp H. J.:* Quantitative comparison of techniques used to measure complement-mediated cytotoxicity of nucleated cells. J. Immunol. *114:* 287–290 1975.
2. *Dickson J. A.:* The uptake of non-metabolizable amino acids as an index of cell viability in vitro. Exp. Cell Res. *61:* 235–245 1970.
3. *Engelfriet C. P. & Britten A.:* The cytotoxic test for leucocyte antibodies. Vox sang. (Basel) *10:* 660–674 1965.
4. *Fleer C. T. G. & Graber I. G.:* Measurement of extracellular fluid volume using ^{131}I labelled inulin. Clin. chim. Acta *8:* 333–342, 1963.
5. *Goldberg B. & Green H.:* The cytotoxic action of immune gamma globulin and complement on Krebs ascites tumor cells. J. exp. Med. *109:* 505–510 1959.
6. *Gorer P. A. & O'Gorman P.:* The cytotoxic activity of isoantibodies in mice. Transplant. Bull. *3:* 142–143, 1956.
7. *Götze O. & Müller-Eberhard H. J.:* Paroxysmal nocturnal hemoglobinuria. Hemolysis initiated by the C3 activator system. New Engl. J. Med. *286:* 180–184 1972.
8. *Okita, G. T., Kabara, J. J., Richardson F. & LeRoy G. V.:* Assaying compounds containing H³ and C¹⁴. Nucleonics *15:* 111–114 1957.
9. *Wigzell, H.:* Quantitative titrations of mouse H-2 antibodies using Cr^{51}-labelled target cells. Transplantation *3:* 423–431 1965.

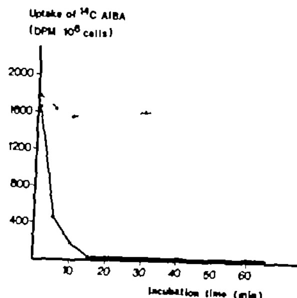


Fig 3 Uptake of ^{14}C-AIBA in sensitized LS-cells in the presence of fresh, respectively inactivated guinea pig complement. ●——● fresh guinea pig serum ▲- - -▲ inactivated guinea-pig serum.

cells were tested for their ability to take up ^{14}C-AIBA

The experiment showed a rapid decline in AIBA uptake after addition of complement and no uptake after incubation for 15 min. Using inactivated GPS the effect of anti serum and complement on AIBA uptake was not seen (Fig 3)

Kinetics of AIBA Efflux from Sensitized Cells in the Presence of Complement

The effects of complement on the efflux of ^{14}C-AIBA from sensitized LS cells were estimated. LS-cells were allowed to equilibrate with medium containing ^{14}C-AIBA and ^{3}H inulin for 90 min after which a cytotoxic dose of inactivated antiserum was added Following a sensitization period of 30 min, fresh GPS was brought into the system and the amounts of intracellular AIBA were measured at varying intervals of time after addition of complement. The medium used in the experiment contained excess of cold AIBA As control served sensitized cells without the addition of complement

The efflux of radioactive AIBA from sensitized LS-cells was greatly accelerated in the presence of complement. Fig 4 illustrates the efflux of ^{14}C-AIBA as function of time in contact with complement.

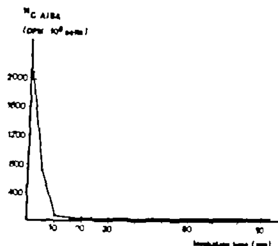


Fig 4 Effect of guinea pig complement on efflux of ^{14}C-AIBA from sensitized LS-cells. The broken line represents sensitized cells without addition of complement.

DISCUSSION

The results described in this paper indicated that in the presence of homologous anti serum and complement, the cells are completely deprived of their ability to accumulate AIBA and also that the efflux of AIBA from prelabelled cells was greatly increased. In the absence of complement, no effect was observed even after prolonged incubation if the dose of antiserum of current interest were used.

The findings indicated serious interference with the mechanisms responsible for transport into the cells, and increased permeability for intracellular constituents.

It is not known whether the increased efflux and reduced uptake of AIBA are due to identical lesions of the cell membrane and it remains also to be shown at which step in the complement sequence the effects occur

The studies undertaken to test the validity of AIBA-uptake as indicator of cell viability and semiquantitation of cytotoxic antibodies in serum showed that the AIBA uptake method had some advantage over the widely used trypan blue test It eliminated some of the variables inherent in the trypan blue test,

TABLE I *The in vitro Effects of Cy lic 3' 5' AMP and Cycli 3' 5'-GMP and their Dib tyryl Derivatives on A H man Leucocyt M gration under Agarose and B. Human LIF Activity*

Drug	Conc. (M)	No. of exp.	A: Migration index	A: P	B: Per cent inhibition of LIF activity	B: P
cAMP	10	3	1.06 (0.97–1 14)	–	4 (17– 28)	–
	10	3	1.0? (0 92–1 15)	–	54 (27 100)	–
	10^{-}	3	0 97 (0.88–1.02)	–	42 (23 60)	–
dibut. cAMP	10^{-7}	5	0.99 ± 0 01†	ns§	39 ± 29†	ns
	10^{-}	5	1.01 ± 0 06	ns	45 ± 22	<0.05
	10^{-}	5	0.97 ± 0.07	ns	50 ± 17	<0.05
	10^{-}	5	1 01 ± 0 09	ns	52 ± 30	<0 05
cGMP	10^{-}	3	1.00 (0.97–1.02)	–	–4 (25–12)	–
	10	3	1.08 (1.00–1 12)	–	16 (–34 54)	–
	10	3	1.09 (0.95–1.23)	–	22 (6–50)	–
dibut cGMP	10^{-}	5	1 15 ± 0 15	ns	–8 ± 24	ns
	10^{-}	5	1 16 ± 0.15	<0 05	12 ± 33	ns
	10	5	1 15 ± 0.23	ns	23 ± 28	ns

Parentheses indicate ranges of three different experiments.
† Standard deviation of five different experiments.
§ P>0 05 was considered not significant (Mann-Whitney rank sum test)

Materials and Methods

The following drugs obtained from Sigma (St. Louis, Mo U.S.A) were used Adenosine 3' 5 cyclic monophosphoric acid (cAMP) N⁶ O²' dibutyryl adenosine 3' 5'-cyclic monophosphoric acid (dibut cAMP) guanosine 3' 5 -cyclic monophosphoric acid (cGMP) and N² O²'-dibutyryl guanosine 3' 5'-cyclic monophosphoric acid (dibut. cGMP) all in the form of sodium salts and of highest purity available

The production and assay of LIF was carried out as previously detailed (1) Briefly 2.5×10^{6} human peripheral blood lymphocytes per ml of serum free, Hepes-buffered culture medium TC-199 were incubated in the presence (LIF-rich) and in the absence (control) of concanavalin A (Con A) 80 μg/ml. Supernatants were depleted of Con A by Sephadex G-100 chromatography and fractions containing molecules between 40 000 and 80 000 daltons were pooled, lyophilized and re dissolved in TC-199 immediately before use Three times concentrated LIF-rich and control supernatants (90 μl) were incubated for 30 min at 37 C, either with a) 10 μl TC-199 as a control for initial lymphokine activity or b) 10 μl of drug solution to give the final concentrations indicated in the table. Venous blood leucocytes (22×10^{6}) were then mixed with each supernatant made to contain 10 per cent v/v horse serum. Subsequently the cells were allowed to migrate under agarose and the migration index (MI) was determined MI = Area of cell migration in LIF-rich supernatant/area of cell migration in control supernatant. The inhibitory effect on LIF was calculated by comparing the MI of supernatants treated with drugs ($MI_{treated}$) with the MI of untreated supernatants ($MI_{control}$) using the formula Per cent inhibition of LIF = ($MI_{treated}$–$MI_{control}$/1 $MI_{control}$) × 100 The effects of the drugs on leucocyte migration were expressed as migration indices calculated as follow MI = Area of cell migration of drug-treated leucocytes/area of cell migration in medium alone.

Result and Discussion

As shown in Table 1 neither cAMP nor dibut. cAMP were found to influence leucocyte migration. Cyclic GMP however seemed to increase the cell motility although the degree of ariation was seen to be remarkably high. As expected, this effect was most pronounced if the dibutyryl derivative of the drug was tested. This product is quite lipid soluble and thus presumed to enter the cells more readily than the parent compound. As further shown in Table 1 cAMP markedly reduced LIF activity (MI ± SD of untreated supernatants 0 66 ± 0 08 n = 13) Again, the effect of the dibutyryl derivative was most pronounced since

Acta path microbiol scand Sect. C 85 78–80 1977

BRIEF REPORT

IN VITRO MODULATION OF HUMAN LEUCOCYTE MIGRATION AND MIGRATION INHIBITORY FACTOR (LIF) ACTIVITY BY CYCLIC 3' 5'-AMP AND CYCLIC 3' 5'-GMP

K Bendtzen and J Palit

Laboratory of Clinical Immunology Medical Department TA, Rigshospitalet University Hospital, Copenhagen, Denmark

Bendtzen K. & Palit J *In vitro* modulation of human leucocyte migration and migration inhibitory factor (LIF) activity by cyclic 3' 5' AMP and cyclic 3' 5'-GMP Acta path. microbiol. scand Sect. C, 85 78–80 1977

The effects of cyclic 3' 5' AMP (cAMP) and cyclic 3' 5'-GMP (cGMP) on the *in vitro* migration of human peripheral blood leucocytes under agarose and on the activity of leucocyte migration inhibitory factor (LIF) was studied. Leucocyte migration was not influenced by dibutyryl cAMP while the dibutyryl derivative of cGMP significantly stimulated cell migration (1×10^{-3}M) LIF-treated leucocytes partially escaped migration inhibition in the presence of dibutyryl cAMP ($\geq 1 \times 10^{-4}$M) while dibutyryl cGMP was inefficient. If the parent compounds cAMP and cGMP were tested almost similar results would be obtained, although at higher concentrations of the drugs. These results represent initial experiments with a view to investigating the possible rôle of cyclic nucleotides in the expression of LIF activity

Key words Human leucocyte migration inhibitory factor (LIF) cyclic AMP cyclic GMP

K. Bendtzen, Laboratory of Clinical Immunology Medical Department TA, Rigshospitalet University Hospital Tagensvej 18 DK 2200 Copenhagen N Denmark.

Received 15.xi.76 Accepted 15.xii.76

Studies of cAMP cGMP and of various drugs known to influence their intracellular levels have led to the conclusion that cyclic nucleotides are involved in the mediation of immune reactions (4 7) Thus, cAMP has been shown to inhibit lymphocyte transformation and to reduce the production of various lymphocyte mediators, lymphokines, such as macrophage migration inhibitory factor (MIF) (7) and leucocyte migration inhibitory factor (LIF) (6) Certain immunological and inflammatory functions of leucocytes and macrophages may also be regulated by cyclic nucleotides, probably by a general inhibitory action of cAMP and a stimulatory action of cGMP (4)

The mechanism by which LIF inhibits leucocyte migration is, on the whole, unknown. Recent experiments, however seem to indicate that LIF may possess an esterase and a protease activity (2) Moreover two esters, α N-benzoyl-L-arginine ethylester and bis-p-nitrophenyl phosphate (BNPP) capable of protecting LIF from inactivation by different serine esterase inhibitors, may function as substrate analogs for the putative LIF enzyme (3) Since the synthetic ester BNPP is capable of substituting as a substrate for phosphodiesterases with 3' nucleotidase activities, the enzymes that degrade cyclic nucleotides (5) it is of great interest to determine whether the action of LIF may decrease the levels of cAMP and/or cGMP in leucocytes. If so, exogeneous cAMP and/or cGMP would be expected to reduce the effect of LIF on leucocytes.

Acta path. microbiol. scand. Sect. C, 85: 81–86, 1977

IMMUNOCHEMICAL CHARACTERISTICS OF SUBFRAGMENTS FROM THE C_H3 HOMOLOGY REGION OF HUMAN IgG

C. Endresen

The Gade Institute, Department of Microbiology, University of Bergen, Bergen, Norway

Endresen, C. Immunochemical characteristics of subfragments from the C_H3 homology region of human IgG. Acta path. microbiol. scand. Sect. C, 85: 81–86, 1977.

A subfragment (peak IV) of F from human IgG belonging to the C_H3 region and found to be slightly different from both pF and tFc fragments was released after tryptic digestion of acid-treated F. Gel filtration at dissociating conditions of reduced and alkylated fragments indicated molecular weight of 10,200. The Fc, pFc, tF and peak IV fragments all interacted with a rheumatoid factor having anti-Gm(1) specificity and showed a reaction of identity in agar diffusion against both anti-F and anti-pFc' sera. In immunoelectrophoresis, peak IV and tFc fragments behaved similarly but with more anodical movement than pFc' fragments. The probable location of the new fragment in terms of the sequenced γ-chain is proposed to be the residues 335 to 439.

Key words: Human IgG; C_H3 homology region.

Curt Endresen, Department of Microbiology, MFH-bygget, N 5016 Haukeland sykehus, Norway.

Received 12.vii.76. Accepted 20.ix.76

Subfragments of the Fc part of human IgG have been prepared by using different proteolytic enzymes and digestion procedures (4, 11, 14, 18, 19). Thus, the human Fc and pFc' fragments were isolated after papain and pepsin digestion respectively (18), the tFc fragment after tryptic digestion of the latter (11).

Upon acid treatment of Fc fragments from human IgG myeloma proteins prior to short exposures to trypsin, *Ellerson et al.* (4) were able to isolate fragments with an antigenicity similar to that of the pFc fragment as well as a fragment containing only the C_H2 region. This method has previously been applied to pooled human Fc, the subfragments being isolated and examined for ability to react with staphylococcal protein A (5). A preliminary examination of the low molecular weight fraction (peak IV) found to be antigenically similar to the pFc fragments (4, 5) revealed an immunoelectrophoretic mobility slightly different from that of pFc.

The purpose of the present investigation was to characterize more closely the fragments of peak IV.

MATERIALS AND METHODS

Human IgG

The Cohn fraction II preparation of pooled human IgG (Kabi AB, Stockholm, Sweden) was used.

F Fragment

IgG was incubated with papain (2 × crystallized from Papaya Latex, Sigma Chemical Co.,

a LIF inhibitory effect of exogenous dibut. cAMP was seen even at concentrations slightly above the physiological intracellular range which is of an order of 10^{-7}M In contrast to this, leucocytes did not escape migration inhibition in the presence of cGMP or its lipid soluble derivative It was only at a very high and unphysiological concentration (1×10^{-4}M) that a slight, but insignificant inhibitory effect was seen.

Although these experiments fail to establish that cyclic nucleotides may play a rôle as intracellular "second messengers" of LIF they demonstrate a clear-cut effect of cAMP on the action of LIF Investigations of the LIF modulating effects of various drugs known to influence the intracellular levels of cyclic nucleotides are currently in progress, and the same applies to direct measurements of the intracellular concentrations of cAMP and cGMP in leucocytes incubated with LIF

The study was supported by grants from the *Danish Medical Research Council* and the *P Carl Petersen Foundation.*

References 1 *Bendtzen K.* Acta Allergol. (Kbh) *30* 327–338 1975.—2 *Bendtzen K.* Acta path microbiol scand. Sect. C, *84* 471–476, 1976.—3. *Bendtzen K.* Scand. J Immunol (in press).—4 *Bourne H R., Lichtenstein L. M., Melmon K. L., Henney C S Weinstein Y & Shearer G M.* Science *184* 19–28. 1974.—5 *Drummond, G I & Yamamoto M* The Enzymes, 3rd ed. (*Boyer P D* (Ed)) Academic Press, New York and London 1971.—6. *Lomnitzer R., Rabson A R. & Koornhof H J* Clin. exp. Immunol. *24* 42–48, 1976.—7 *Pick E.* Current Titles Immunol. Transpl. Allerg. *4* 597–601 1976

Acta path. microbiol. scand. Sect. C, 85: 81-86, 1977

IMMUNOCHEMICAL CHARACTERISTICS OF SUBFRAGMENTS FROM THE C_H3 HOMOLOGY REGION OF HUMAN IgG

C. Endresen

The Gade Institute, Department of Microbiology, University of Bergen, Bergen, Norway

Endresen, C. Immunochemical characteristics of subfragments from the C_H3 homology region of human IgG. Acta path. microbiol. scand. Sect. C, 85: 81-86, 1977.

A subfragment (peak IV) of Fc from human IgG belonging to the C_H3 region and found to be slightly different from both pFc' and tFc' fragments was released after tryptic digestion of acid-treated Fc. Gel filtration at dissociating conditions of reduced and alkylated fragments indicated a molecular weight of 10,200. The Fc, pFc', tFc' and peak IV fragments all interacted with rheumatoid factor having anti-Gm(1) specificity and showed a reaction of identity in agar diffusion against both anti-Fc and anti-pFc' sera. In immunoelectrophoresis, peak IV and tFc' fragments behaved similarly but with a more anodical movement than pFc' fragments. The probable location of the new fragment in terms of the sequenced γ-chain is proposed to be the residues 335 to 439.

Key words: Human IgG; C_H3 homology region.

Curt Endresen, Department of Microbiology, MFH-bygget, N-5016 Haukeland sykehus, Norway

Received 12.xi.76 Accepted 20.xi.76

Subfragments of the Fc part of human IgG have been prepared by using different proteolytic enzymes and digestion procedures (4, 11, 14, 18, 19). Thus, the human Fc and pFc' fragments were isolated after papain and pepsin digestion respectively (18), the tFc' fragment after tryptic digestion of the latter (11).

Upon acid treatment of Fc fragments from human IgG myeloma proteins prior to short exposures to trypsin, *Ellerson et al.* (4) were able to isolate fragments with an antigenicity similar to that of the pFc' fragment as well as a fragment containing only the C_H2 region. This method has previously been applied to pooled human Fc, the subfragments being isolated and examined for ability to react with staphylococcal protein A (5). A preliminary examination of the low molecular weight fraction (peak IV) found to be antigenically similar to the pFc' fragments (4, 5) revealed an immunoelectrophoretic mobility slightly different from that of pFc'.

The purpose of the present investigation was to characterize more closely the fragments of peak IV.

MATERIALS AND METHODS

Human IgG

The Cohn fraction II preparation of pooled human IgG (Kabi AB, Stockholm, Sweden) was used.

Fc Fragments

IgG was incubated with papain (2 × crystallized from Papaya Latex, Sigma Chemical Co.,

a LIF inhibitory effect of exogenous dibut. cAMP was seen even at concentrations slightly above the physiological intracellular range which is of an order of 10^{-7}M. In contrast to this, leucocytes did not escape migration inhibition in the presence of cGMP or its lipid-soluble derivative. It was only at a very high and unphysiological concentration (1×10^{-4}M) that a slight but insignificant inhibitory effect was seen.

Although these experiments fail to establish that cyclic nucleotides may play a rôle as intracellular "second messengers" of LIF they demonstrate a clear-cut effect of cAMP on the action of LIF. Investigations of the LIF modulating effects of various drugs known to influence the intracellular levels of cyclic nucleotides are currently in progress, and the same applies to direct measurements of the intracellular concentrations of cAMP and cGMP in leucocytes incubated with LIF.

The study was supported by grants from the *Danish Medical Research Council* and the *P. Carl Petersen Foundation*.

References: 1 *Bendtzen, K.:* Acta Allergol. (Kbh.) *30:* 327–338, 1975.—2 *Bendtzen, K.:* Acta path. microbiol. scand. Sect. C, *84:* 471–476, 1976.—3 *Bendtzen, K.:* Scand. J. Immunol. (in press).—4 *Bourne, H. R., Lichtenstein, L. M., Melmon, K. L., Henney, C. S., Weinstein, Y. & Shearer, G. M.:* Science *184:* 19–28, 1974.—5 *Drummond, G. I. & Yamamoto, M.:* The Enzymes, 3rd ed. (*Boyer, P. D.* (Ed.)). Academic Press, New York and London, 1971.—6 *Lomnitzer, R., Rabson, A. R. & Koornhof, H. J.:* Clin. exp. Immunol. *24:* 42–48, 1976.—7 *Pick, E.:* Current Titles Immunol. Transpl. Allerg. *4:* 597–601, 1976.

Amino Acid Analysis

Samples (1 2 mg) of fragments were hydrolysed in 6 N HCl at 110 C for 24 48 and 72 h and analyzed by gas liquid chromatography (16) Corrections for loss during hydrolyzation were made by extrapolation to zero-time hydrolysis.

N-terminal Amino Acids

were examined by both the dansylation method (20) and by the use of leucine amino peptidase (Sigma) (9) using 0.05–0 1 μmole quantities of protein. Aliquots were removed from the digest after varying time intervals The maximum incubation time tested was 96 h Free amino acids were analyzed by thin-layer chromatography (20) and gas liquid chromatography (16)

C-terminal Amino Acids

were analyzed using Carboxypeptidase A (Sigma) (1) Protein samples (0 05–0.1 μmole) were digested with enzyme (substrate to enzyme ratio, 40 1) up to 48 h at 37 C. Aliquots were removed at differing incubation times and tested for free amino acids.

RESULTS

Double diffusion analysis showed complete fusion of the lines formed between the pFc tFc and the peak IV fragments against both anti-Fc and anti-pFc sera (Fig 1) In immunoelectrophoresis, however a difference in

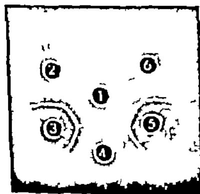


Fig. 1 Double diffusion in agar gel of peak IV (1) pF (2 and 6) and tF (4) fragments against rabbit anti-human pF (3) and rabbit anti-human F sera (5)

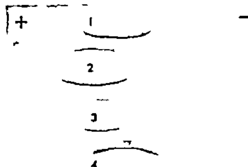


Fig 2 Immunoelectrophoresis in agar of pFc (1) tF (2) peak IV fragment (3) and Fc fragments (4) Rabbit anti-human pFc serum in the middle trough, and rabbit anti-human F serum I the upper and lower troughs.

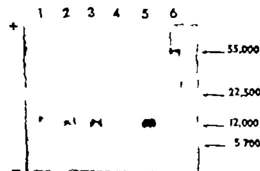


Fig 3 SDS polyacrylamide gel electrophoresis of pFc (1) tFc (2) and peak IV fragments (3) Insulin (4) Cytochrome C (5) and reduced and alkylated IgG (6)

electrophoretic mobility was observed between the pFc' fragment and the tFc' and the peak IV fragments (Fig 2) When the digestion of pFc' with trypsin was stopped after 2 h, the precipitation line appeared to be more heterogeneous, indicating the presence of both tFc and pFc fragments.

SDS polyacrylamide gel electrophoresis showed that the peak IV fragments moved similarly to cytochrome C, indicating a molecular weight of the monomer chains of about 12,000 (Fig. 3) On gel filtration at dissociating conditions (5 M guanidine-1 M HAc) the fragments were eluted at sym-

USA) for 16 h in 0.1 M phosphate buffer pH 7.0 containing 0.01 M EDTA Na_2, the papain substrate ratio being 1:100 (w/w). Proteolysis was first performed without cysteine (12). The digest was gelfiltered through a Sephadex G-150 column (3.2 × 88 cm) in 0.1 M Tris-HCl, pH 7.8 containing 0.2 M NaCl, 2 mM EDTA Na_2 and 0.05 per cent Na azide. The Fc and Fab fragments were separated by chromatography on DEAE-cellulose columns according to *Rossi & Nisonoff* (17). All preparations were checked for purity by double diffusion in agar and immunoelectrophoresis using rabbit antisera to human serum, Fc and Fab fragments. The isolated Fc fragments were further digested with papain as described above, this time with cysteine present (12). Homogeneous Fc fragments were isolated after gel filtration on Sephadex G-100 columns equilibrated with 0.01 M Tris-HCl buffer pH 7.8 with 0.15 M NaCl.

Fragmentation of Purified Fc Fragments

Fc fragments were treated with acid and trypsin (Type XI DCC treated, Sigma) (4) and the digest gelfiltered through a Sephadex G-100 column equilibrated with 1 N HAc (4, 5).

After re-cycling of the low-molecular weight fraction through a column (2.5 × 55 cm) of Sephadex G-50 superfine in 0.01 M Tris-HCl buffer pH 7.8 containing 0.15 M NaCl, the main fraction was concentrated and used as purified peak IV fragments.

Preparation of pFc' Fragments

A pepsin digest of 300 mg human IgG was gel filtered through a Sephadex G-150 column as described in (18).

Preparation of tFc' Fragments

Purified pFc' fragments were digested with trypsin (11). After 4 h the digestion was stopped with trypsin inhibitor and the digest gelfiltered through a Sephadex G-50 superfine column in 0.01 M Tris-HCl buffer pH 7.8 containing 0.15 M NaCl. The major fractions in a similar elution position to that of the parent pFc' fragment were concentrated and referred to as tFc' fragments; the purity was examined by immunoelectrophoresis.

Apparent Molecular Weight

The fragments were reduced and alkylated (13) and apparent molecular weights determined according to *Andrews* (2) using the K_a values (7) of the gelfiltered proteins on a column (1.6 × 74.1 cm) of Sephadex G-100 in 5 M guanidine-1 M HAc. The column was calibrated with reduced and alkylated human IgG (Kabi AB), cytochrome C (Koch-Light, England) and insulin (Sigma). Molecular weights were also estimated using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) according to *Fairbanks et al.* (6). The electrophoresis was performed in a Bio-Rad Model 220 apparatus, the gel contained 11.2 per cent acrylamide and 1 per cent SDS, and the pH of the Tris-sodium acetate buffer used was 7.4.

Antisera

to human serum, IgG and Fc fragments were obtained from rabbits injected in the foot pads with saline solutions of antigen emulsified with an equal volume of complete Freund's adjuvant (Difco, Mich., USA). Two booster injections (the second 10 days after the first) were given subcutaneously, beginning 8 weeks after the first injection. Blood was drawn from the marginal ear vein 6–7 days after each booster injection. An anti pFc' serum was kindly provided by Dr. *T. E. Michaelsen*, Institute of Immunology and Rheumatology, The University Hospital, Oslo, Norway.

Immunoelectrophoresis

was performed using an LKB apparatus (LKB Produkter, Sweden) with special Agar Noble (Difco) in barbitone buffer (I 0.05, pH 8.6) and a voltage of 6–8 V/cm.

Double Diffusion in Agar

was carried out as described by *Ouchterlony* (15) in 1 per cent special Agar Noble (Difco) in barbitone buffer (I 0.025, pH 8.6).

Haemagglutination Inhibition

Human O Rh+ (R_1R_2) erythrocytes were sensitized with anti D/Gm (1) serum (Behringwerke, Germany) (8), washed and suspended in saline to a 0.5 per cent concentration. Haemagglutination inhibition tests were performed in perspex microtitrator trays (Takatsy, Hungary). Two-fold dilutions (25 µl volume) of the IgG fragments were mixed with the same volume of anti-Gm (1) containing 4 agglutinating units and then incubated at 37° C for 45 min. Twenty-five microlitres of the suspension of sheep erythrocytes sensitized with anti D/Gm (1) antibodies were then added, the mixtures were stored at room temperature for 2 h, and then at 4° C overnight. The lowest concentration of antigen which inhibited the agglutination is referred to as the inhibiting concentration.

Concentration and Quantification of Proteins

Protein solutions were concentrated by ultrafiltration using Amicon Cells and UM 2 filters (Diaflo, Oosterhout, The Netherlands) and concentrations determined as described in (10).

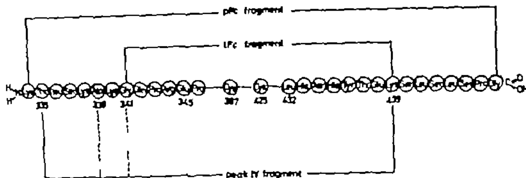


Fig. 5. The proposed location of the peak IV in the C_H3 homology region compared to pFc' and tFc' fragments (11). The peak IV fragments, like the pFc' and tFc' fragments, are assumed to be dimeric according to the gel filtration experiments (11, 14). The amino acid residues are numbered in the manner used for the Eu protein (3).

well with the results obtained earlier (11, 14, 18). Both the chemical and the enzymatic methods indicated that glycine is the main N-terminal amino acid of the tFc' fragments (11, 18). The N-terminal amino acid analyses of the peak IV fragments revealed that some heterogeneity was apparent at the amino terminus. However, threonine was found to be the main N-terminal amino acid, but alanine, glycine and glutamic acid was also detected.

The leucine and serine content of both peak IV and tFc' fragments indicates that these fragments have lost a short peptide (heptapeptide) from the C-terminal end. According to the amino acid sequence of the Eu protein (3), two leucine and three serine residues are included in the γ-chain between lysine at position 439 and the C-terminal glycine at position 446 (11).

Using Carboxypeptidase A, a considerable amount of glycine was split off after short digestion of pFc' fragments. In contrast, only traces of glycine together with small amounts of lysine were digested. This indicates the presence of lysine at position 439 of peak IV (Fig. 5), as was previously found for tFc' (11). *Turner et al.* (19), however, found that the tF' fragments had lost only five amino acid residues from the N-terminal, as compared to pFc'. C-terminal glycine being found in both subfragments.

The N-terminal amino acids found in peak IV fragments suggests that short trypsin treatment after exposure to acid splits the γ-chain at differing positions between residue 334 and 345 (Fig. 5). This conclusion was also supported by the amino acid analysis, which revealed slight differences between peak IV and tFc' fragments.

The gel filtration (Fig. 4) indicates that the peak IV fragments (K_{av} = 0.43) are somewhat smaller than the tFc' fragments (K_{av} = 0.41, mol.wt. 10,800).

The slight difference in size of the peak IV and tFc' fragments seem to be related to the substrate used. When pFc' fragments were digested with trypsin (45 sec) after exposure to acid (4), tFc' like fragments were produced (K_{av} = 0.41), but when intact IgG was exposed to acid and then digested with trypsin (45 sec), peak IV fragments K_{av} = 0.43) were produced (personal observation). The Gm (1) marker earlier found on the pFc', tFc' and other subfragments of the C_H3 homology region (14, 19), was also shown to be present on the peak IV fragments. However, the marker was apparently better expressed in the Fc and pFc' fragments than in the peak IV and in the tFc' fragments (Table 3).

Subfragments lacking larger pieces of the C-terminal part, including the asparagine residue 434, have lost their ability to react with anti-Gm (1) antibodies (14, 19). Therefore, it has been proposed that the asparagine residue should be defined as a non-correlative

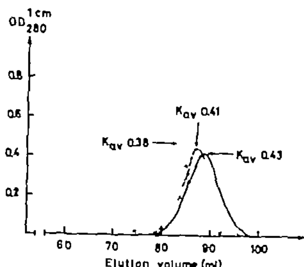


Fig 4 Gel filtration on Sephadex G-100 of reduced and alkylated C_{II}3 fragments. Column size 1 6 × 74 1 cm V_0 = 45.5 ml sample volume 0.5 ml; sample concentration 4–6 mg/ml elution rat 2.55 ml per hour eluant 5 M guanidine–1 M acetic acid. pFc (xxx) tFc (– – –) peak IV (——)

metrical peaks (Fig. 4) The K_{av} of pFc was 0 38 (mol.wt. 11,800) K_{av} of tFc 0 41 (mol wt. 10,800) and that of peak IV fragments 0 43 (mol.wt. 10,200)

TABLE 1 Amino Acid Composition (Moles/Mole Protein) of the Fragments

Amino acid	Fragment		
	pFc	tFc	peak IV
Tryptophan	N.D	N.D	N.D
Lysine	17.5	14 7	14.3
Histidine	N.D	N.D	N.D
Arginine	N.D	N.D	N.D
Aspartic acid	21 7	21 1	22.3
Threonine	14 9	14 3	13.3
Serine	?6 7	20 1	19 4
Glutamic acid	?7 0	27 0	27 0
Proline	15 1	12 6	13 7
Glycine	12 6	10 7	9 9
Alanine	5 4	4 9	6 1
Valine	15 6	15 4	15 0
Methionine	3 1	2 7	2.3
Leucine	17 4	13 7	13.5
Isoleucine	2 9	3 0	? 3
Tyrosine	8 2	6 1	7.3
Phenylalanine	7 4	6 4	6.9
Cysteine	N.D	N.D	N.D

N.D – not determined.

TABLE 2 *Analysis of Terminal Amino Acids of pFc' tFc' and Peak II Fragments*

Fragment	Predominant N-terminal residue	Predominant C-terminal residue
pFc	Lysine	Glycine
tFc	Glycine	Lysine
peak IV	Threonine	Lysine

TABLE 3 Results of Haemagglutination Inhibition Test

Fragment	Concentration of fragments (mg/ml) inhibiting the agglutination between anti-Gm (1) antibodies and human O red blood cells coated with anti-D/Gm (1) antibodies
Fc	0 125–0 250
pFc	0.250
tFc	0.5
peak IV	0.5
Fab	>10 0

The results of the quantitative amino acid analysis are shown in Table 1 and the N and C-terminal amino acids of the various fragments in Table 2. Table 3 shows the results of inhibition of the haemagglutination by Fc fragments, its various subfragments, and Fab fragments. In contrast to Fab, which served as a negative control, both Fc and all subfragments inhibited the agglutination system.

DISCUSSION

The various subfragments behaved identically in double diffusion but the fragments moved differently in immunoelectrophoresis, in agreement with the earlier observation on tFc and the pFc fragments (11) The peak IV fragments and the tFc fragments moved more anodically than the pFc fragments, probably due to a smaller amount of lysine (Table 1)

The amino acid composition of the pFc and the tFc fragments and their terminal amino acids found in the present study fit

Acta path. microbiol. scand. Sect. C, 85 87-89 1977

FREQUENCY OF IgA DEFICIENCY IN BLOOD DONORS AND Rh NEGATIVE WOMEN IN ICELAND

SIGURDUR GUDMUNDSSON and OLAFUR JENSSON

The Blood Bank, Reykjavík, Iceland

Gudmundsson, S & Jensson, O Frequency of IgA deficiency in blood donors and Rh negative women in Iceland. Acta path. microbiol scand. Sect. C, 85 87-89 1977

Sera from 6,842 individuals were tested for IgA deficiency using double and radial immunodiffusion Sera containing less than 1 mg/100 ml of IgA were classified as deficient. The frequency of selective IgA deficiency among 4 799 blood donors investigated was 1:533 but 1:340 among 1,017 Rh negative women screened and 1:485 for both groups combined. One of the nine IgA deficient blood donors detected belonged to a 1st cousin marriage family previously investigated, in which the mother also was deficient in IgA. One IgA deficient recipient was found among 704 hospital patients screened for this abnormality

Key words IgA deficiency frequency blood donors Rh negative women 1st cousin marriage

S. Gudmundsson, The Blood Bank, Reykjavík, Iceland.

Received 13 ix 76 Accepted 30 76

A preliminary report on screening of Icelandic blood donors for IgA deficiency was read at the ISBT congress in Helsinki 1975 (7) The screening was continued in 1975 and the results are reported in this paper

MATERIAL AND METHODS

Double immunodiffusion. Ouchterlony (5) was used and 1 per cent agarose (L'Industrie Biologique Francaise S.A. Gennevilliers, France) dissolved in 0 15 M phosphate buffered NaCl, pH 7.2 Test serum used was rabbit anti-IgA from Behringwerke AG Standard Human Serum (Behringwerke Batch N 974) used contained IgA = 250 mg/100 ml = 149 I U/100 ml. Umbilical cord sera were also used as control

Radial immunodiffusion was used for quantification, using Immuno-Plate Human IgA (1 wells) Hyland Lab.

These methods will detect quantities down to approx. 1 mg/100 ml of IgA. More sensitive methods necessary to estimate IgA levels less than 1 mg/100 ml (3) have not been used. In this survey individuals in whom IgA was undetectable by the methods described, were classified as IgA deficient.

RESULTS

The results of screening 6,842 individuals are recorded in Table 1 The frequency of IgA deficiency is 1:533 in the donor group and 1:340 among the Rh negative women investigated. When the two groups are combined the frequency of IgA deficiency is 1:485.

Over 90 per cent of the blood donors tested were males 18-60 years of age. The Rh negative women investigated were in the age group 16-45 years and blood samples for the tests were collected after three months of pregnancy in most of the cases and after six months in other cases.

One individual among 704 hospital pa

determinant responsible for the conformation of the Gm (1) marker (19) Thus, the poorer expression of the Gm (1) marker found in the tFc and peak IV fragments, both having lost a heptapeptide from the C-terminal end can be due to small changes in the conformation

REFERENCES

1 *Ambler P P* Enzymatic hydrolysis with carboxypeptidases. In *Hirs C H W & Timasheff S N* (Eds.) Methods in Enzymology vol XXV part B Academic Press, New York and London 1972, pp 143–154

2 *Andrews P* Estimation of the molecular weights of proteins by Sephadex gel filtration. Biochem J *91* 222–233 1964

3 *Edelman G M Cunningham B A Gall P D Rutishauser U & Waxdal M J* The covalent structure of an entire γG immunoglobulin molecule Proc. nat Acad Sci. (Wash) *63* 78–85 1969

4 *Ellerson J R Yasmeen D Painter R H & Dorrington K J* A fragment corresponding to the C_H2 region of immunoglobulin G (IgG) with complement fixing activity FEBS Letters *24* 318 322 1972

5 *Endresen C Heggeness M & Grov A* Tryptic fragments of Fc from normal human IgG and their interaction with staphylococcal protein A. Scand. J Immunol *3* 261–267 1974

6 *Fairbanks G Steck T L & Wallach D F H* Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry *10* 2606–2617 1971

7 *Laurent T C & Killander J* A theory of gel filtration and its experimental verification J Chromatogr *14* 317 330 1964

8. *Lawler S D* Genetic studies on γ globulin with particular reference to man. In *Weir D M* (Ed) Handbook of Experimental Immunology *Blackwell Scientific Publications*, Oxford 1967 pp 329–352

9 *Light A* Leucine aminopeptidase in sequence determination of peptides. In *Hirs C H W & Timasheff S N* (Eds.) Methods in Enzymology vol. XXV part B. Academic Press, New York and London 1972, pp. 253–262.

10 *Lowry O H Rosebrough N J Farr A L & Randall R J.* Protein measurement with Folin phenol reagent. J biol. Chem. *193* 265–275 1951

11 *Matthews N Stewart G & Stanworth D R* Characterization of a new tryptic subfragment of human and rabbit IgG Fc fragment Immunochemistry *8* 973–981 1971

12. *Michaelsen T E & Natvig J B.* Isolation and characterization of IgG subclass proteins and Fc fragments from normal human IgG Immunochemistry *8* 235–242 1971

13 *Michaelsen T E & Natvig J B.* Three new fragments, $F(ab)_2$ $F(c)_2$ and Fab/c obtained by papain proteolysis of normal human IgG Scand. J Immunol. *1* 255–268, 1972.

14 *Okafor G O & Turner M W* Immunochemical characteristics and rheumatoid factor inhibiting activity of enzymic subfragments from Cγ3 homology region of human IgG Scand. J Immunol *3* 181–190 1974

15 *Ouchterlony O* Diffusion-in-gel for immunological analysis. Progr Allergy *5* 1–78, 1958.

16 *Roach D & Gherke C W* The gas-liquid chromatography of amino acids. J Chromatogr *43* 303–310 1969

17 *Rossi G & Nisonoff A* Crystallization of fragment Fab of human IgG myeloma protein Biochem. biophys. Res. Commun. *31* 914–918 1968

18 *Turner M W & Bennich H* Subfragments from the Fc fragment of human immunoglobulin G Isolation and physicochemical characterization. Biochem J *107* 171–178, 1968.

19 *Turner M W Komvopoulos A Bennich H & Natvig J B* Antigenic and immunological characteristics of tryptic and chymotryptic subfragments from the Cγ3 homology region of human Immunoglobulin G Scand J Immunol *1* 53–62 1972

20 *Zanetta J P Vincendon G Mandel P & Combos G* The utilisation of 1-dimethylaminonaphtaline-5-sulphonyl chloride for quantitative determination of free amino acid and partial analysis of primary structure of proteins. J Chromatogr *51* 441–458, 1970.

FREQUENCY OF IgA DEFICIENCY IN BLOOD DONORS AND Rh NEGATIVE WOMEN IN ICELAND

SIGURDUR GUDMUNDSSON and ÓLAFUR JENSSON

The Blood Bank, Reykjavík, Iceland

Gudmundsson, S. & Jensson, O. Frequency of IgA deficiency in blood donors and Rh negative women in Iceland. Acta path. microbiol. scand. Sect. C, 85: 87–89, 1977.

Sera from 6,842 individuals were tested for IgA deficiency using double and radial immunodiffusion. Sera containing less than 1 mg/100 ml of IgA were classified as deficient. The frequency of selective IgA deficiency among 4,799 blood donors investigated was 1:533, but 1:340 among 1,017 Rh negative women screened and 1:485 for both groups combined. One of the nine IgA deficient blood donors detected belonged to a 1st cousin marriage family previously investigated, in which the mother also was deficient in IgA. One IgA deficient recipient was found among 704 hospital patients screened for this abnormality.

Key words: IgA; deficiency; frequency; blood donors; Rh negative women; 1st cousin marriage.

S. Gudmundsson, The Blood Bank, Reykjavík, Iceland.

Received 15.ix.76. Accepted 30.x.76.

A preliminary report on screening of Icelandic blood donors for IgA deficiency was read at the ISBT congress in Helsinki 1975 (2). The screening was continued in 1975 and the results are reported in this paper.

MATERIAL AND METHODS

Double immunodiffusion, Ouchterlony (5) was used and 1 per cent agarose (L'Industrie Biologique Française S.A. Gennevilliers, France) dissolved in 0.15 M phosphate buffered NaCl, pH 7.2. Test serum used was rabbit anti-IgA from Behringwerke AG. Standard Human Serum (Behringwerke, Batch N 974) used contained IgA = 150 mg/100 ml = 149 I.U./100 ml. Umbilical cord sera were also used as control.

Radial immunodiffusion was used for quantification, using Immuno-Plate Human IgA (1 wells) Hyland Lab.

These methods will detect quantities down to approx. 1 mg/100 ml of IgA. More sensitive methods necessary to estimate IgA levels less than 1 mg/100 ml (3) have not been used. In this survey individuals in whom IgA was undetectable by the methods described, were classified as IgA deficient.

RESULTS

The results of screening 6,842 individuals are recorded in Table 1. The frequency of IgA deficiency is 1:533 in the donor group and 1:340 among the Rh negative women investigated. When the two groups are combined the frequency of IgA deficiency is 1:485.

Over 90 per cent of the blood donors tested were males 18–60 years of age. The Rh negative women investigated were in the age group 16–45 years and blood samples for the tests were collected after three months of pregnancy in most of the cases and after six months in other cases.

One individual among 704 hospital pa-

TABLE 1 *Results of Screening for IgA Deficiency in Iceland*

Types of population	Number of individuals tested	Number of IgA deficient individuals	Frequency
1 Blood donors (Mainly males)	4 799	9*	1/533
2. Rh negative women (Pregnant)	1 017	3	1/340
1 + 2	5 816	12	1/485
3 Recipients (Hospital patients)	704	1	1/704
4 1st cousin marriage (Family members)	322	2	
Total	6,842	15	

* One of the IgA deficient donors belongs to a 1st cousin marriage family

TABLE 2 *A First Cousin Marriage Family Containing Two Members with Selective IgA Deficiency*

		Ig mg/100 ml					Ig subtypes Gm a–c⁵												Inv 1 a		Am 12	
		E‡	G	A	M	D	a	x	f	n	g	b^0	b^1	b^3	s	t	c^3	c^5	1	a	1	2
01*	09	18 0	1600 0	360 0	106.0	3 0	+	+	+	+	+	+	+	+	—	—	—	—	—	—	+	—
02	24	15 0	3360 0	0 0	160 0	0 0	+	+	—	—	+	—	—	—	—	—	—	—	—	—		
12	48	44 0	2480 0	180 0	19[illegible]0	5 0	+	+	+	+	+	+	+	+	+	—	—	—	—	—	+	—
41	56	26 0	1320 0	0 0	54 0	0.0	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—
51	62	38.0	1600 0	180 0	192.0	7 0	+	—	+	+	+	+	+	+	+	—	—	—	—	—	+	—

* The blood donor (4156) and his mother (0224) are deficient in IgA Column 1 = position (0 = parents) 2 = sex (1 = male 2 = female) 3-4 = year of birth.

‡ The Ig main classes were determined by Dr *D R Stanworth* University of Birmingham, England and Ig subtyping (Gm Inv and Am) was done by Dr *Erna van Loghem* Central Laboratory of Blood Transfusion Amsterdam, Holland.

tients (recipients) tested was found to be deficient in IgA One of the IgA deficient donors detected belongs to a 1st cousin marriage family previously investigated The results on five members of this family are shown in Table 2

DISCUSSION

The frequency of selective IgA deficiency has been found to vary from 1 310–1 3 040 (1 3) depending on the subjects selected and the sensitivity of the method used (3) In a large survey of 64,588 donors in Finland, the frequency of selective IgA deficiency was 1.396 using double diffusion (DD) as screening method (3) In Canada the frequency of selective IgA deficiency among 15,500 Rh negative women screened by DD was found to be 1 1 300 (6) or much lower than that among Rh negative Icelandic women (Table 1) The size of the sample of Icelandic blood donors screened is over 2 per cent of the total population (216 000 in December 1975)

No transfusion reaction due to anti-IgA (7) has been diagnosed in Iceland up to the present time. However it is considered of value to keep a record of IgA deficient individuals detected and thereby single out a proportion of individuals at risk when receiving blood and plasma fractions (4 6 7) In addition such record will make IgA deficient blood donors available for patients manifesting reaction due to anti-IgA.

The first cousin marriage family investigation referred to was supported financially by the *Atomic Energy Commission* (AEC) Contract No AT(30-1) 3548 to The Genetical Committee of The University of Iceland.

REFERENCES

1. *Frommel, D. Moullec J., Lambin P & Fine J H* Selective serum IgA deficiency Vox Sang. 25 513–518, 1973.
2. *Gudmundsson S & Jensson O* XIV Congress of the International Society of Blood Transfusion, Helsinki, 1975
3. *Koistinen J* Studies of Selective Deficiency of Serum IgA and its Significance in Blood Transfusion. Academic dissertation, Helsingin YLIOPISTON Monistuspalvelu Offset, 1975.
4. *Leikola J Koistinen J Lehtinen, M & Virolainen M.:* IgA-induced anaphylactic transfusion reactions A report of four cases. Blood 42 111–119 1973
5. *Ouchterlony O* Handbook of Immunodiffusion and Immuno-electrophoresis, Ann Arbor Science Publishers Inc. Michigan U.S.A. 1–51 1973
6. *Pai M K R., Davison M., Bedritis I & Zipursky A.*. Selective IgA deficiency in Rh-negative women. Vox Sang. 27 87 91 1974
7. *Vyas G N & Fudenberg, H H.:* Anaphylactoid transfusion reactions associated with anti-IgA. Lancet II 312 315 1968.

Acta path. microbiol. scand Sect. C, 85 90–98 1977

COMPARISON OF MICROTITRE PLATES WITH FLAT-BOTTOMED AND ROUND-BOTTOMED WELLS FOR MIXED LYMPHOCYTE CULTURE (MLC)

EIJA HERVA

Public Health Laboratory Oulu, Finland

Herva, E. Comparison of microtitre plates with flat-bottomed and round bottomed wells for mixed lymphocyte culture (MLC) Acta path. microbiol. scand. Sect. C, 85 90–98, 1977

To compare microtitre plates with flat-bottomed and round-bottomed wells and to standardize a method for mixed lymphocyte culture (MLC) the effects of cell number culture time ^{3}H thymidine concentration and labelling time were studied. On both plates, allogeneic cells induced increased RNA synthesis beginning at about 30 hours and increased DNA synthesis beginning at about 50 hours, if suitable cell numbers were used. On plates with flat-bottomed wells 1.5×10^5 responding and stimulating cells per well had near-exponential growth on day four and five, often through day six on plates with round-bottomed wells the corresponding cell number was 0.25–1 0 (optimally 0.5) $\times 10^5$ Near these cell numbers, the response depended closely on the number of responding cells. On plates with flat-bottomed wells, stimulating cells had a dose-dependent effect on the response whereas on plates with round-bottomed wells, increasing the stimulating cell dose did not consistently strengthen the response. Both plate types proved suitable for MLC experiments plates with round bottomed wells have the obvious advantage of requiring smaller cell numbers. ^{3}H-thymidine (spec. act 2000 mCi/mmol) self-suppressed its incorporation, which increased only slightly or even decreased if labelling time exceeded 12–18 hours. Relative responses remained virtually unaltered, however with ^{3}H-concentrations of 0 5 and 2.0 μCi/ml and with labelling times of 8 and 24 hours.

Key words Mixed lymphocyte culture microtitre plates.

Eija Herva Public Health Laboratory Kajaanintie 46 D 90100 Oulu 10 Finland.

Received 27.ix.76 Accepted 11.x.76

The mixed lymphocyte culture (MLC) reaction (*Bain et al* 1964 *Bach & Hirschhorn* 1964) representing the sensitizing part of allograft reaction in vitro (*Häyry & Defendi* 1970) has found wide use as a tool in clinical histocompatibility testing and in basic immunological research (for discussion and reference of technical and biological aspects of MLC, see *Sorensen* 1972 *Häyry et al* 1972 *Thorsby* 1974a *Eijsvoogel* 1974)

MLC microtechniques based on the measurement of DNA synthesis (*Hartzman et al* 1971 *Seagar & Terasaki* 1971 *du Bois et al* 1973) were compared in the Oslo Workshop in March 1974 (*Thorsby* 1974b)

In this paper microtitre plates with flat bottomed and round-bottomed wells are com

pared in regard to the MLC reaction profiles and to the effects of cell number and radioactive labelling conditions on the results. The participants of the Oslo Workshop recommended clarification of these points in each laboratory using the MLC technique (*Thorsby et al* (1974)

MATERIALS AND METHODS

Preparation of Mononuclear Cells

Venous blood (from healthy laboratory personnel unless otherwise specified) was aseptically drawn into glass tubes or plastic syringes containing heparin (Heparin Medica Medica Pharmaceuticals, Helsinki, Finland) 25–50 units per ml of blood. The mononuclear cells were separated from undiluted blood within 4 hours according to the method described by *Bøyum* (1968) washed 2 3 times with Hanks BSS and suspended in Medium 199 (Orion Diagnostica, Helsinki, Finland) containing 20 per cent AB serum from healthy males. Both solutions were supplemented with 100 units of penicillin-G and 100 μ of streptomycin per ml. Medium 199 contained, in addition, 1.25 mg of L-glutamine and 0.29 mg of sodium bicarbonate per ml. The cell suspensions were kept in tissue culture tubes at 10° C and used within 24 hours.

Trypan blue staining has shown the viability of the cells to be 95–100 per cent. The mononuclear cell yield has been about 70 per cent in our laboratory (*Rykbhen* 1975)

Irradiation of Stimulating Cells

Cell suspensions, at a concentration of 1.0–2.0 × 10⁶/ml, were exposed to a 3000 rad dose of roentgen radiation (250 kV HV 7.2 mmCu, 40 rad/min) (*Kasakura & Lowenstein* 1965 *Hirva & Kuusisto* 1975) in tissue culture tubes at room temperature immediately before preparation of cultures.

MLC Micro-technique

The cultures were prepared according to modifications (*Bo d uk et al* 1974 *Jørgensen & Lamm* 1974) of the miniature technique described by *Hartzman et al* (1971) with some minor differences in the use of apparatus.

Preparation of Cultures

Each combination was tested in triplicate or quadruplicate cultures containing 0.1 ml of both component cell suspensions per culture (plates with flat-bottomed wells Falcon Plastics, cat. no. 3040, lid 3041 Calif., USA or M220-29 ART C.A. Greiner et Söhne West Germany; round-bottomed wells M24 ARTL, Sterilin Ltd., Richmond, Surrey England) A Hamilton dispenser (PB-600-10 Hamilton Switzerland) with a 5 ml glass syringe was used for the distribution. The syringe was rinsed with distilled water at least five times between different cell suspensions. For control each cell preparation was tested against irradiated autologous cells. Most experiments also included control combinations of irradiated cells some included control wells with plain medium. Excessive alkalisation of the cell suspensions during preparation of the cultures was prevented by bubbling carbon dioxide into the cell suspension tubes when necessary. Generally 0.2 ml of water was placed into peripheral wells of the plate to prevent dehydration.

Incubation and Radioactive Labelling

The cultures were incubated in a CO_2-incubator (Forma Scientific Inc., Ohio USA) with a humidified atmosphere of 5 per cent CO_2 in air at 37 C for various lengths of time. Unless otherwise specified, 2 μl of ³H-thymidine (Methyl-³H thymidine code TRA.310 spec. act. 2000 mCi/mmol The Radiochemical Centre Amersham, England) in physiological saline was added 24 hours before harvest into each well using a Hamilton dispenser (PB-600 Hamilton, Switzerland) to get a final concentration of 2.0 μCi/ml. The ³H-uridine (5-³H-uridine code TRA.178 spec. act 2000–5000 mCi/mmol, The Radiochemical Centre) used in some experiments was added 12 hours before harvest for a final concentration of 2.5 μCi/ml.

Harvesting and Scintillation Counting

The cultures were washed onto glass fiber filters (Type A or E, part. no. 61638 Gelman Instr Co Ann Arbor Mich., USA) using a multiple cell culture harvester (Skatron AS, Lierbyen, Norway) Filter sheets were dried at 80 C for an hour. Each filter disc, corresponding to one microculture, was placed into a glass scintillation vial (Vidox Jena) or plastic scintillation counter insert vial (Product no. 505 Sterilin Ltd Richmond, Surrey England) Scintillation fluid (Permablend III Packard Instr Co Inc., III USA 5.5 g/litre of toluene) was then added 3 ml (or 2 ml, which turned out to be sufficient) into the vial or 1 ml into the insert vial. The radioactivity was measured in a LKB Wallac (Turku, Finland) liquid scintillation counter for 5 minutes.

Calculation and Expression of the Results

The results are expressed as the mean and standard deviation of the triplicate or quadruplicate cpms, without subtracting the background. In some cases, the results are also given as the median.

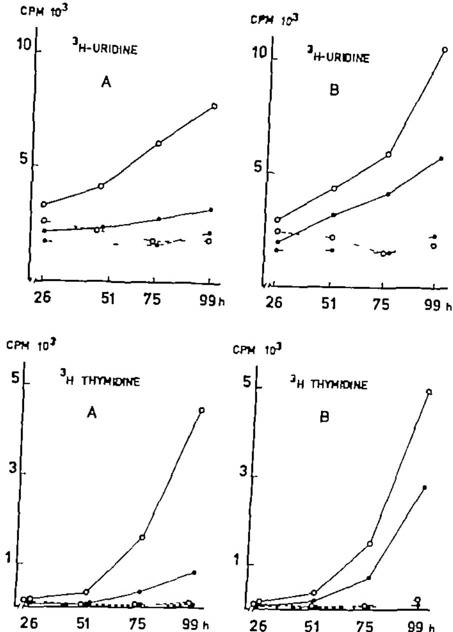


Fig 1 RNA and DNA synthesis in mixed lymphocyte cultures (MLC) 0.75×10^5 (●—●) or 1.5×10^5 (○—○) *responding cells were cultured with the same number of irradiated allogeneic cells in* flat-bottomed (A) or round bottomed (B) wells of microtitre plates for various lengths of time. ^{3}H uridine or H thymidine were added 12 hours before harvest. Dashed lines control cultures "stimulated" with autologous irradiated cells. Each point represents the cpm of triplicate cultures.

RESULTS

1 *MLC Reaction Profiles*

In cultures in round bottomed wells and also in flat bottomed wells with the higher cell number (1.5×10^5 responding and stimulating cells per well) significantly increased ^{3}H uridine incorporation (RNA synthesis) and ^{3}H thymidine incorporation (DNA synthesis) was seen at 51 hours. Slightly increased RNA synthesis occurred already at 26 hours (Fig 1) In flat bottomed wells, the lower cell number (0.75×10^5) reacted later and the reaction was proportionately weaker

At about 70 hours near-exponential growth (DNA synthesis) began continuing for two or three days. Increasing DNA synthesis

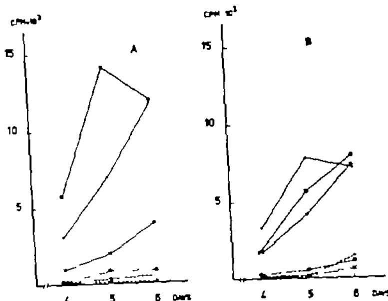


Fig 2 Effect of cell number and type of culture plate on MLC response. 0 75 (×—×) 1.5 (●—●) or 3.0 (○—○) × 10⁶ responding and stimulating cells were cultured on microtitre plates with flat bottomed (A) or round-bottomed (B) wells and harvested on arious days. Each point represents the mean cpm quadruplicate cultures. Dashed lines utologous control cultures.

lasted to day five or six, sometimes even to day seven, depending to some extent on the number of cells per culture. After the peak response, DNA synthesis declined rapidly (not illustrated)

2. *MLC Reaction with Various Numbers of Responding and Stimulating Cells in a Ratio 1 1*

In flat-bottomed wells, optimal cell number was 1.5×10^5 responding and stimulating cells per well (Fig 2A) A lower cell number ($0\ 75 \times 10$) gave a proportionately weaker reaction when calculated per number of cultured cells. Doubling the cell number to 3.0×10^5 of each component per well gave a double reaction, if harvested on day four or five but not if harvested on day six.

Fig. 2B shows that in round-bottomed wells $0\ 75 \times 10^5$ responding and stimulating cells reacted twice as strongly as in flat-bottomed wells. With 1.5×10^5 of both cells per well, this difference was no longer seen. 3.0×10^5 of both cells were evidently too many giving a weaker reaction than 1.5×10 cells. Exponential growth from day four to day six was seen only with $0\ 75 \times 10^5$ responding and stimulating cells per culture.

A similar experiment to that illustrated in Fig. 2 was also performed with a MLC-combination reacting three times as strongly Again, 1.5×10 responding and stimulating cells per culture in flat-bottomed wells and 0.75×10^5 of both cells in round-bottomed wells were found optimal.

Fig 3A shows that in round-bottomed wells 0.25 0.50 and 1.0×10 responding and stimulating cells gave a reaction roughly dependent on cell number These and other experiments demonstrated that 0.50×10^5 of both cells per well reacted usually most strongly per cultured cell number

Thus, on microtitre plates with flat-bottomed wells, about 1.5×10^5 responding and stimulating cells were required to get a cell-number dependent reaction. On plates with round-bottomed wells this was attained with

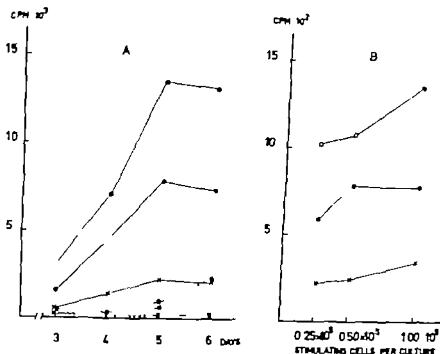


Fig 3A Effect of cell number on MLC response on microtitre plates with round-bottomed wells The results of an one-way experiment with 0.25 (×——×) 0.50 (●——●) or 1 0 (○——○) × 10^3 responding and stimulating cells per culture are shown. Each point represents the mean of triplicate cultures. Dashed lines autologous control cultures.

Fig 3B Effect of stimulating cell dose on MLC response on microtitre plates with round-bottomed wells. Results of an experiment with 0 25 (×——×) 0.50 (●——●) or 1 0 (○——○) × 10^3 responding cells per culture and various numbers of irradiated stimulating cells are shown the cultures were harvested on day 5 Each point represents the mean cpm of triplicate cultures. Autologous control cultures have been omitted from the figure.

0.50 × 10^3 (or even 0.25 × 10^3) of both cells. More than 1.5 × 10^3 in flat bottomed wells and more than 0 75 × 10^3 in round bottomed wells of each cell population tend to diminish the response per cultured cell number especially if harvested after day five.

3 *Effect of Stimulating Cell Dose on MLC Reaction*

On plates with flat-bottomed wells, increasing the stimulating cell dose strengthened the response very clearly (Fig 4A) The response was most directly dependent on the stimulating cell dose when there were 1 5 × 10^3 responding cells per culture On plates with round bottomed wells the effect of stimulating cell dose was weaker (Fig 4B) These findings were supported by a corresponding experiment with a cell combination reacting about three times more strongly

When smaller responding cell numbers (0 25 –1 0 × 10^3) were used in round-bottomed wells, the MLC reaction was found to depend closely on the number of responding cells. The effect of stimulating cell dose was weak and variable (Fig 3B)

4 *Effect of Labelling Conditions on MLC Reaction Result*

Lengthening the labelling time up to eight hours increased H thymidine incorporation sharply but further lengthening induced no substantial increase in the incorporation, regardless of the concentration of ^{3}H thymidine (Fig 5A) Other experiments (not illustrated) showed that the highest incorporation was seen in cultures *harvested after 12–18* hours labelling

Increasing the ^{3}H thymidine concentration from 0.5 to 1 0–2 0 μCi/ml increased its in

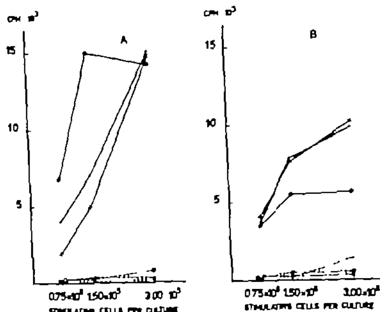


Fig. 4. Effect of stimulating cell dose on MLC response. 0.75 (×—×), 1.5 (●—●) or 3.0 (○—○) × 10⁵ responding cells per culture were stimulated with various numbers of irradiated cells on microtitre plates with flat-bottomed (A) or round-bottomed (B) wells; the cultures were harvested on day 5. Each point represents the mean cpm of triplicate cultures. Dashed lines: autologous control cultures.

corporation sharply. Further increase had a less pronounced effect (Fig. 5B). The effect of ³H-thymidine concentration was similar in both plate types (not illustrated).

In spite of the observed effects of labelling conditions on the results, similar relative responses (*Jorgensen & Lamm* 1974) were obtained with various ³H-thymidine concentrations and labelling times (Fig. 6). For routine practice, the most convenient labelling time of 24 hours and a ³H-thymidine concentration of 2.0 μCi/ml were chosen.

5. *Variation between Multiple Cultures*

In the experiments presented in this paper the coefficient of variation among cultures made in triplicate or quadruplicate was, on the average, 8–20 per cent in MLC positive and 20–50 per cent in MLC negative and autologous combinations.

DISCUSSION

Suitable conditions for the MLC reaction are determined by two factors: sufficient reciprocal contact between the cells and sufficient medium environment to ensure their growth (*Moorhead et al.* 1967, *du Bois et al.* 1973).

In flat-bottomed wells, adequate intercellular contact is achieved either by using a fairly large number of responding and stimulating cells or by increasing the stimulating cell dose. With equal numbers of responding and stimulating cells, 1.5 × 10⁵ of both per well was found to be optimal. Around this cell number a response most closely dependent on the numbers of both cells was obtained, and exponential growth was observable on days four and five and often on day six.

We have not studied whether the discriminatory power of the method or its general applicability can be improved by increasing

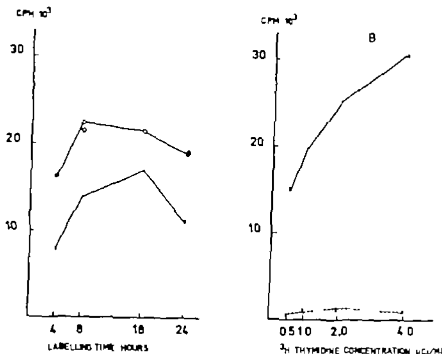


Fig 5 Effect of ^{3}H-thymidine concentration and labelling time on incorporation of radioactivity in MLC. 5A The one-way MLC experiments in round-bottomed wells with 0.5×10^5 responding and stimulating cells were harvested on day 5 ^{3}H-thymidine (spec. act. 2000 mCi/mmol) was added 4 8, 18 or 24 hours before harvest for a final concentration of 0.5 µCi/ml (●—●) or 2.0 µCi/ml (O—O) The points connected with lines represent the mean cpm of triplicate cultures, the points without lines are the median cpm values. 5B The one-way MLC experiments in round-bottomed wells with 0.75×10^5 responding and stimulating cells per culture were harvested on day 5 various amounts of ^{3}H thymidine were added 18 hours before harvest. The mean and median cpm values are shown (see above)

the stimulating cell dose but there are observations suggesting that certain weak responses can thus be distinguished from a negative MLC result (*Albertini* & *Bach* 1968)

Our results in MLC experiments made on plates with flat bottomed wells are very similar to those reported by other groups (*Hartman et al* 1971 *Bondevik et al.* 1974)

In round bottomed wells the cells have sufficient reciprocal contact and the crowding effect (*du Bois et al* 1973) limits growth at smaller cell numbers than in flat-bottomed wells. 0.5×10^5 responding and stimulating cells gave the strongest response per number of cultured cells and had an exponential growth curve from day three to day five.

Stimulating cells did not have a dose dependent effect on the response in round bottomed wells, contrary to the observations made using plates with flat bottomed wells. In this particular respect, our results differ from those reported by *Jorgensen* & *Lamm* (1974) who reported a linear increase with stimulating dose increases. It is possible that our stimulating cells are more resistant and cause optimal stimulation even in small doses. We used the irradiated cells immediately and our radiation dose was smaller viz. 3000 rad *Jorgensen* & *Lamm* kept the irradiated (5000 rad) cells overnight or preserved them frozen. Radiation damage is known to advance over time though considerably more slowly at temperatures lower than at 37° C (*Vaughan Smith* & *Ling* 1974)

When optimal cell numbers (in ratio 1 1 of responding and stimulating cells) were used, the response per cultured cell number was usually stronger in round bottomed wells than in flat-bottomed ones. The difference

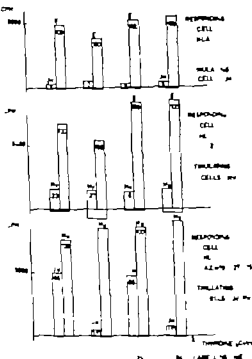


Fig. 6. Effect of H-thymidine concentration and labelling time on the discriminatory capacity of the MLC test. Cells from patient with multiple sclerosis (M) typing cells (J homozygous for HLA-Dw2) and control cells (E) were cultured on microtitre plates with round-bottomed wells (0.5 × 10⁴ responding and stimulating cells per culture) for 5 days. ³H-thymidine (spec. act. 2000 mCi/mmol) was added 8 or 24 hours before harvest for a final concentration of 0.5 or 2.0 µCi/ml. Each column represents the mean cpm of triplicate cultures expressed as specific response or increment (the mean cpm of allogeneic cultures minus the mean cpm of autologous cultures). The mean cpm values of triplicate autologous cultures are shown under the baseline. The responses are also expressed as relative responses (figures in columns) with the greatest response of each responding cell taken as 100. (TF) = technical failure.

was not great, however, and in some experiments the responses were identical. Similar observations were reported by *du Bois et al.* (1974) who however consistently obtained stronger responses using round-bottomed plates. When an excess (2:1 or 4:1) of stimulating cells was used (with a sufficiently low number of responding cells) the response per responding cell number was even stronger in flat-bottomed wells than in round-bottomed wells.

On both plate types, cultures with suitable cell numbers are best harvested, and their results compared, on day five during near exponential growth (*Sørensen* 1972).

The problems associated with the expression of the results of MLC experiments were thoroughly discussed in the Oslo Workshop (see *Thorsby et al.* 1974).

I thank Mrs *Raili Marttukauppi* and *Kaarina Jääskeläinen* for their skillful technical assistance, and Miss *Raili Liukko* for her help in drawing the figures and typing the manuscript.

REFERENCES

Albertini, R. J. & Bach, F. H.: Quantitative assay of antigenic disparity at HL-A—the major histocompatibility locus in man. J. Exp. Med. *128*: 639–651, 1968.

Bach, F. H. & Hirschhorn, K.: Lymphocyte interaction: A potential histocompatibility test in vitro. Science *142*: 813–814, 1964.

Bain, B., Vas, M. R. & Lowenstein, L.: The development of large immature mononuclear cells in mixed leukocyte cultures. Blood *23*: 108–116, 1964.

Bondevik, H., Helgesen, A., Thorsby, A. B. & Thorsby, E.: A mixed lymphocyte culture micro-technique and its application in different family and unrelated combinations. Tissue Antigens *4*: 469–481, 1974.

Bøyum, A.: Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest. 21, suppl. *97*: 77–89, 1968.

Du Bois, M. J. G. J., Huismans, D. R., Schellekens, P. Th. A. & Eijsvoogel, V. P.: Investigation and standardization of the conditions for micro-lymphocyte cultures. Tissue Antigens *3*: 402–409, 1973.

Du Bois, R., Meinesz, A., Bierhorst-Eijlander, A., Groenewoud, M., Schellekens, P. Th. A. & Eijsvoogel, V. P.: The use of microtiter plates in mixed lymphocyte cultures. Tissue Antigens *4*: 458–468, 1974.

Eijsvoogel, V. P.: The cellular recognition in vitro of antigens related to human histocompatibility. Semin. Hematol. *11*: 305–324, 1974.

Hartzman, R. J., Segall, M., Bach, M. L. & Bach, F. H.: Histocompatibility matching. VI. Miniaturization of the mixed leukocyte culture test:

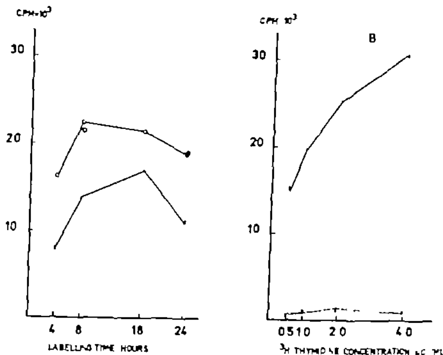


Fig 5 Effect of ³H thymidine concentration and labelling time on incorporation of radioactivity in MLC. 5A The one-way MLC experiments in round-bottomed wells with 0.5×10^5 responding and stimulating cells were harvested on day 5 ³H thymidine (spec. act. ?000 mCi/mmol) was added 4 8, 18 or 24 hours before harvest for a final concentration of 0.5 µCi/ml (●—●) or 2.0 µCi/ml (○—○) The points connected with lines represent the mean cpm of triplicate cultures, the points without lines are the median cpm values. 5B The one-way MLC experiments in round-bottomed wells with 0.75×10^5 responding and stimulating cells per culture were harvested on day 5 various amounts of ³H thymidine were added 18 hours before harvest. The mean and median cpm values are shown (see above)

the stimulating cell dose but there are observations suggesting that certain weak responses can thus be distinguished from a negative MLC result (*Albertini & Bach* 1968)

Our results in MLC experiments made on plates with flat bottomed wells are very similar to those reported by other groups (*Hartman et al* 1971 *Bondevik et al* 1974)

In round-bottomed wells the cells have sufficient reciprocal contact and the crowding effect (*du Bois et al.* 1973) limits growth at smaller cell numbers than in flat bottomed wells. 0.5×10^5 responding and stimulating cells gave the strongest response per number of cultured cells and had an exponential growth curve from day three to day five.

Stimulating cells did not have a dose-dependent effect on the response in round bottomed wells, contrary to the observations made using plates with flat-bottomed wells. In this particular respect, our results differ from those reported by *Jorgensen & Lamm* (1974) who reported a linear increase with stimulating dose increases. It is possible that our stimulating cells are more resistant and cause optimal stimulation even in small doses. We used the irradiated cells immediately and our radiation dose was smaller viz. 3000 rad *Jorgensen & Lamm* kept the irradiated (5000 rad) cells overnight or preserved them frozen. Radiation damage is known to advance over time though considerably more slowly at temperatures lower than at 37 C (*Vaughan-Smith & Ling* 1974)

When optimal cell numbers (in ratio 1 1 of responding and stimulating cells) were used, the response per cultured cell number was usually stronger in round-bottomed wells than in flat bottomed ones. The difference

Acta path. microbiol. scand. Sect. C, 85 99–106 1977

MIXED LYMPHOCYTE CULTURE REACTIONS BETWEEN PARENTAL CELLS IN PREGNANCY AND PUERPERIUM

Eija Herva and Pentti Jouppila

Public Health Laboratory Oulu and Department of Obstetrics and Gynecology University of Oulu, Finland

Herva, E. & Jouppila, P. Mixed lymphocyte culture reactions between parental cells in pregnancy and puerperium. Acta path. microbiol. scand Sect. C, 85 99–106, 1977

Mixed lymphocyte culture (MLC) reactions of maternal cells *with paternal and control cells*, inhibition of these reactions with maternal serum, and cytotoxic antibodies in maternal serum were studied in 36 couples through pregnancy and puerperium. Average MLC reactions of maternal cells with paternal and control cells were not significantly different, nor did the MLC responses of maternal cells clearly differ from those of other cells in their time-course kinetics. However the response of maternal cells to paternal cells was weaker than to control cells in 15 of 18 couples studied during the first week postpartum; this may suggest some specific alteration in maternal immunity to paternal *antigens. The sera of pregnant women suppressed* MLC reactions. This suppressive effect disappeared during the first week postpartum, whereafter only about 20 per cent of the sera studied caused clear inhibition of MLC reactions. Nine per cent of the postpartum sera studied contained cytotoxic antibodies against paternal cells.

Key words Mixed lymphocyte culture reactions; parental cells; pregnancy puerperium.

Eija Herva, Public Health Laboratory Kajaanintie 46 D 90100 Oulu 10, Finland.

Received 27.ix.76 Accepted 11.x.76

Pregnant females, human mothers included, are often immunized against fetal transplantation antigens during pregnancy Nevertheless, the fetus seems never or almost never to be rejected by the mother and maternal immunization against fetal antigens may be rather a benefit than a complication to the fetus. Immunological hyporeactivity of the mother non-expression of transplantation antigens in fetal tissues, and an immunologically inert barrier between maternal and fetal tissues have been proposed as possible mechanisms to explain the nonrejection of the fetus (for discussion and reference see *Anderson* 1971 *Beer* & *Billingham* 1971 *Billington* 1975 Lancet 1975)

Mixed lymphocyte culture (MLC) reaction (*Bain et al.* 1964 *Bach* & *Hirschhorn* 1964) represents the sensitizing phase of allograft reaction in vitro (*Häyry* & *Defendi* 1970) Its strength depends on the degree of histoincompatibility between the cell donors (for discussion and reference, see *Sorensen* 1972, *Thorsby* 1974)

MLC reaction is weakened by early thymectomy and abolished specifically by induced tolerance. Immunization against major histocompatibility complex (MHC) antigens may cause a specifically strengthened and

7*

A preliminary report. Transplantation *11* 268–273 1971

Herva E & Kiviniitty K The effect of in vitro irradiation on the responses of human lymphocytes to PHA, PPD and allogeneic cells. Strahlentherapie *149* 504–512 1975

Häyry P & Defendi V Mixed lymphocyte cultures produce effector cells An in vitro model for allograft rejection. Science *168* 133–135 1970

Häyry P Andersson L C Nordling, S & Virolainen M Allograft response in vitro. Transplant. Rev *12* 91–140 1972

Jorgensen F & Lamm L U MLC- a micromodification of the mixed leukocyte culture technique. Tissue Antigens *4* 482–494 1974

Kasakura S & Lowenstein L The effect of irradiation in vitro on mixed leukocyte cultures and on leukocyte cultures with phytohaemagglutinin. In *Balner H Cleton S J & Eernisse J G* (Eds.) Histocompatibility Testing 1965 Munksgaard Copenhagen 1965 p 211–212

Moorhead J F Connolly J J & McFarland W Factors affecting the reactivity of human lymphocytes in vitro I Cell number duration of culture and surface area. J Immunol. *99* 413–419 1967

Rykänen P The effects of anaesthesia- and surgical stress on immune response in different age groups. In Abstracts of the Postgraduate Course and Congress Papers. The Twelfth Congress of the Scandinavian Society of Anaesthesiologists, Oulu, Finland July 7–12, 1975 p. 55–56

Sengar D P S & Terasaki P I A semimicro mixed leukocyte culture test. Transplantation *11* 260–267 1971

Sørensen F S The mixed lymphocyte culture interaction. Techniques and immunogenetics Acta path. microbiol. scand. Sect. B. Suppl. 230 1972

Thorsby E The human major histocompatibility system. Transplant. Rev *18* 51–129 1974a

Thorsby E. (Ed) Reports from a mixed lymphocyte culture workshop Tissue Antigens *4* 455–525 1974b.

Thorsby E du Bois R Bondevik H Dupont B. Eijsvoogel V Hansen J A Jersild C., Jorgensen F Kissmeyer Nielsen F., Lamm, L U Schellekens P Th. A., Svejgaard A. & Thomsen M Joint report from a mixed lymphocyte culture workshop. Tissue Antigens *4* 507–525 1974

Vaughan-Smith S & Ling, N C The effect of X rays on the response of porcine lymphocytes to the mitogenic stimulus of concanavalin A in vitro Int. J Radiat. Biol. *25* 73–85 1974

TABLE 1 *MLC Response of Maternal Cells to Paternal and Control Cells in Pregnancy and Puerperium*

	1st trimester	2nd trimester	3rd trimester	1st wk postp.	1st and 2nd mo postp.
MF_x (number of experim.)	18621 ± 9431§ (30)	17776 ± 8856 (17)	13569 ± 9499 (20)	13158 ± 7973 (18)	15079 ± 10355 (14)
MC_x (number of exprim.)	18826 ± 8238 (30)	19594 ± 10911 (17)	14609 ± 10505 (21)	15897 ± 6759 (18)	15453 ± 5361 (14)
Significance of difference	NS†	NS	NS	NS	NS
Ratio $\frac{MF_x \text{ (mean)}}{MC_x \text{ (mean)}}$	0.99	0.91	0.93	0.83	0.98
$\frac{MF_x}{MC_x}$ <1þ	16/30	8/17	13/20	15/18	7/14

M = maternal cells responding F_x = paternal, C = control cells stimulating.
§ The mean ± SD values of each cell combination have been calculated from the increment (MF_x–MM_x, MC_x–MM_x) cpm values of cultures harvested on day six.
† NS = not significant (Student t-test)
þ MF_x/MC_x ratio calculated for each experiment separately

maternal serum diluted in equal volume of AB-serum)

Cultures were made in triplicate, occasionally in duplicate or quadruplicate and harvested on day six, often also on day four

For calculation and presentation of MLC results the specific responses (increments the mean CPM of triplicate allogeneic cultures minus the mean CPM of triplicate autologous cultures) were used Background was not subtracted from the CPM values The occasional experiments with signs of obvious technical failures were excluded.

Student t-test was used to evaluate the statistical significance of the differences.

Cytotoxic Antibodies

Complement-dependent cytotoxic antibodies in maternal sera against paternal cells were screened using two-stage microcytotoxicity method (*Amos et al* 1969)

RESULTS

MLC Reactivity of Maternal Cells

Average MLC responses of maternal cells to paternal cells did not significantly differ from their responses to unrelated control cells in any phase of pregnancy or puerperium (Table 1) Also the individual MLC responses of maternal cells to paternal cells were weaker than the responses to control cells in about half of the experiments during pregnancy and in late puerperium. However during the first postpartum week, the response to paternal cells was weaker in 15 of the 18 experiments (Table 1 bottom)

Maternal cells stimulated paternal cells less than did control cells from the second trimester of pregnancy through the puerperium (Table 2) However control cells were similarly stimulated less by maternal than by paternal cells. The responses of maternal cells to paternal and control cells were also weaker than the responses of paternal and control cells to each other The above differences were not, however statistically significant.

Maternal lymphocytes preincubated at 37 C overnight responded and stimulated less vigorously than cells stored at 10 C in four of the six experiments performed, but the effect was only slight. In one experiment, preincubated cells responded and stimulated clearly better than those stored at 10 C. Thus, the differences described in the preceding paragraph may be partly due to technical causes.

accelerated MLC response in animals (for discussion and reference see *Sorensen* 1972) and also in man (*Bondevik* & *Thorsby* 1974)

At delivery mother's cells respond in MLC to the cells of her newborn baby (*Ceppellini et al* 1971) and the reaction produces cytotoxic lymphocytes (*Bonnard* & *Lemos* 1972) MLC response of mother's cells to baby's cells is, however weaker than the response of father's cells to baby's cells, the genetic distance being the same in both situations (*Ceppellini et al* 1971) Some mothers are clearly hypo- or unresponsive to their infants cells (*Ceppellini et al* 1971 *Jenkins* & *Good* 1972 *Herva* 1976)

There are also observations suggesting that in pregnancy a woman's cells respond more weakly to her husband's cells than to the cells of an unrelated control person (*Lewis et al.* 1966 *Ceppellini et al* 1971) This difference is more pronounced in multiparous than in primiparous women (*Jenkins* & *Hancock* 1972)

Pregnant women's sera contain nonspecific factors (*Kasakura* 1971 *Leikin* 1972) inhibiting MLC and other in vitro responses of lymphocytes. In addition they may contain specific MLC-inhibiting antibodies (*Leventhal et al* 1970) In many of the previous studies, the role of maternal serum in the altered pattern of MLC reactions has not been considered thus impairing the conclusions.

We have studied the possible modifications of maternal lymphocyte responsiveness in MLC against paternal lymphocytes and the effect of maternal serum on MLC reactions during pregnancy

MATERIALS AND METHODS

Subjects

Thirty-six unselected women attending two municipal antenatal clinic in Oulu and their husbands were studied. The age of the mothers varied between 19 and 37 years (mean, 25 years) Nineteen of them had not given births 16 had one full term delivery In both groups, five had had one abortion No obstetrical history was obtained from one mother

During the study all but one of the mothers gave birth to fullterm babies one mother had an abortion at the end of the first trimester

Protocol of the Study

Mixed lymphocyte culture experiments between the cells from mother (M) father (F) and control (C a healthy unrelated male usually) were performed in each trimester of pregnancy during the first week after delivery and one to two months postpartum. To minimize the effect of maternal serum on cellular reactivity the mothers cells were incubated overnight at 37 C in a medium containing 20 per cent pooled normal AB-serum, and the medium was changed before culturing. The corresponding maternal serum sample was studied each time in separate cultures. In addition, a serum sample taken at delivery was studied in cultures during the first postpartum week. Maternal serum from these cultures was diluted with equal amounts of AB-serum to decrease the effect of nonspecific inhibitors thus the final concentration of the serum being tested in the culture was 10-12 per cent.

Cytotoxic antibodies in maternal serum against paternal cells were studied together with the MLC experiments in addition, a large part of collected frozen-stored serum samples were tested again in the postpartum period

Nineteen of the couples were studied at three to five times during pregnancy and puerperium in 17 couples, only one or two MLC experiments were performed successfully

Preparation of Mononuclear Cells

Venous blood samples were collected into sterile glass tubes or plastic syringes containing 25-30 units heparin (Medica Pharmaceuticals, Helsinki, Finland) per milliliter of blood. Mononuclear cells were prepared usually within six hours using the gradient centrifugation method (*Boyum* 1968) stored at 10 C in medium containing 5 per cent AB-serum (maternal cells before delivery see above) and cultured within 30 hours. Maternal sera for MLC inhibition and cytotoxicity studies were separated from venous blood samples without anticoagulant.

MLC Experiments

A modification (*Bondevik et al.* 1974) of the mixed lymphocyte culture micromethod described by *Hartzman et al.* (1971) was used as reported (*Herva* 1977) The experiments were made on flat-bottomed microtitre plates with 1.5×10^5 responding and irradiated stimulating cells per well in a total volume of 0.25 ml of Medium 199 containing 20–24 per cent heat-inactivated serum (AB-serum from healthy non-transfused males, or

TABLE 1 *MLC Response of Maternal Cells to Paternal and Control Cells in Pregnancy and Puerperium*

	1st trimester	2nd trimester	3rd trimester	1st wk postp.	1st and 2nd mo postp.
MF_X (number of experim.)	18621 ± 9451§ (30)	17776 ± 8856 (17)	13569 ± 9499 (20)	13158 ± 7973 (18)	15079 ± 10355 (14)
MC_X (number of experim.)	18826 ± 8238 (30)	19594 ± 10911 (17)	14609 ± 10505 (21)	15897 ± 6759 (18)	15453 ± 5361 (14)
Significance of difference	NS†	NS	NS	NS	NS
Ratio $\frac{MF_X \text{ (mean)}}{MC_X \text{ (mean)}}$	0.99	0.91	0.93	0.83	0.98
$\frac{MF_X}{MC_X} < 1$♭	16/30	8/17	13/20	15/18	7/14

M = maternal cells responding. F_X = paternal, C = control cells stimulating.

§ The mean ± SD values of each cell combination have been calculated from the increment (MF_X–MM_X, MC_X–MM_X) cpm values of cultures harvested on day six.

† NS = not significant (Student' t-test)

♭ MF_X/MC_X ratio calculated for each experiment separately

maternal serum diluted in equal volume of AB-serum)

Cultures were made in triplicate, occasionally in duplicate or quadruplicate and harvested on day six, often also on day four

For calculation and presentation of MLC results the specific responses (increments: the mean CPM of triplicate allogeneic cultures minus the mean CPM of triplicate autologous cultures) were used. Background was not subtracted from the CPM values. The occasional experiments with signs of obvious technical failures were excluded.

Student t-test was used to evaluate the statistical significance of the differences.

Cytotoxic Antibodies

Complement-dependent cytotoxic antibodies in maternal sera against paternal cells were screened using two-stage microcytotoxicity method (*Amos et al.* 1969)

RESULTS

MLC Reactivity of Maternal Cells

Average MLC responses of maternal cells to paternal cells did not significantly differ from their responses to unrelated control cells in any phase of pregnancy or puerperium (Table 1) Also, the individual MLC responses of maternal cells to paternal cells were weaker than the responses to control cells in about half of the experiments during pregnancy and in late puerperium. However during the first postpartum week, the response to paternal cells was weaker in 15 of the 18 experiments (Table 1 bottom)

Maternal cells stimulated paternal cells less than did control cells from the second trimester of pregnancy through the puerperium (Table 2) However control cells were similarly stimulated less by maternal than by paternal cells. The responses of maternal cells to paternal and control cells were also weaker than the responses of paternal and control cells to each other The above differences were not, however statistically significant.

Maternal lymphocytes preincubated at 37 C overnight responded and stimulated less vigorously than cells stored at 10 C in four of the six experiments performed, but the effect was only slight. In one experiment, preincubated cells responded and stimulated clearly better than those stored at 10° C. Thus, the differences described in the preceding paragraph may be partly due to technical causes.

TABLE 2 *MLC Response of Paternal Cells to Maternal and Control Cells in Pregnancy and Puerperium*

	1st trimester	2nd trimester	3rd trimester	1st wk postp	1st and 2nd mo postp.
FM_x* (number of experim.)	23024 ± 12568§ (30)	16502 ± 7988 (17)	14183 ± 7382 (20)	13369 ± 5959 (17)	16349 ± 640[illegible] (12)
FC_x (number of exprim)	21700 ± 19042 (30)	23624 ± 7553 (17)	18177 ± 6996 (21)	16107 ± 6220 (18)	21121 ± 6745 (12)
Significance†	NS	+	NS	NS	NS
Ratio $\frac{FM_x}{FC_x}$	1 06	0 70	0 78	0 83	0.77

* F = paternal cells responding M_x = maternal, C_x = control cells stimulating.

§ The mean ± SD values have been calculated from the increment (FM_x–FF_x, FC_x–FF_x) cpm values of cultures harvested on day six.

† NS = not significant + = almost significant, $p<0.05$ (Student's t test)

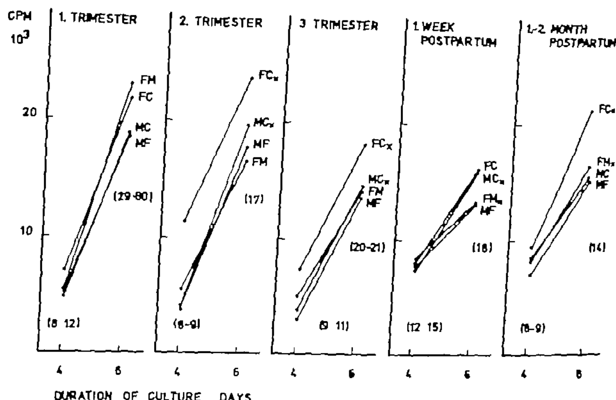


Fig 1 Time-course kinetics of MLC reactions during pregnancy and puerperium M = maternal, F = paternal cells responding M_x F_x, C_x = maternal paternal and control cells stimulating. () = number MLC combinations studied

Time course of Maternal MLC Response

Time-course curves of maternal MLC responses were usually similar to those of other responses, and the responses of maternal cells to paternal and control cells usually had similar course (Fig 1 for clarity the responses of control cells have been omitted) In the first postpartum week, however the reactions of maternal and paternal cells with each other were lower on day six than the other reactions.

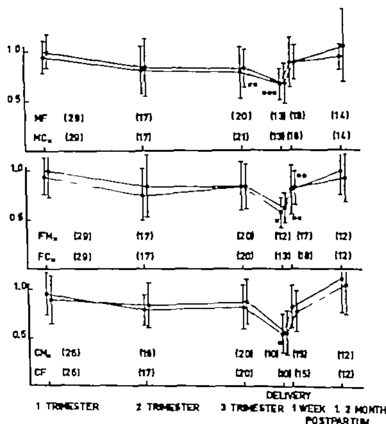


Fig. 2 Effect of maternal serum on MLC reaction during pregnancy and puerperium. The results are presented as the mean ± SD ratio of the responses in the presence of maternal and control serum. For explanations, see Fig. 1 x = almost significant ($p < 0.05$) xx = significant ($p < 0.01$) xxx = highly significant ($p < 0.001$)

MLC Inhibiting Effect and Cytotoxic Antibodies in Maternal Serum

Maternal serum suppressed MLC reactions from the second trimester of pregnancy to the first postpartum week (Fig. 2) Sera taken at delivery caused the strongest suppression, the effect weakening markedly during the first week postpartum.

Twenty-three of the couples were studied in the postpartum period. Sera from three of these mothers markedly inhibited (to under 0.50 of control values) the responses of maternal cells to paternal cells, and also other MLC reactions studied. Sera from two additional mothers had a weaker suppressive effect.

In two of the three cases with marked post partum MLC inhibition, cytotoxic antibodies to paternal cells were sought on four occasions in pregnancy and the postpartum period. The serum of one mother was cytotoxic in the second trimester reacted equivocally in the third trimester and was negative post partum. The other mother's serum was not cytotoxic from the first trimester to the first week postpartum.

Of the two mothers having weaker MLC inhibiting sera, one's serum was weakly cytotoxic to paternal cells in the first week post partum, but negative after two months the other's serum was strongly cytotoxic in the first postpartum week and also a month later

TABLE 2 *MLC Response of Paternal Cells to Maternal and Control Cells in Pregnancy and Puerperium*

	1st trimester	2nd trimester	3rd trimester	1st wk postp.	1st and 2nd mo postp.
FM_x* (number of experim.)	23024 ± 12568§ (30)	16502 ± 7988 (17)	14183 ± 7382 (20)	13369 ± 5959 (17)	16349 ± 6409 (12)
FC_x (number of exprim.)	21700 ± 12042 (30)	23674 ± 7553 (17)	18177 ± 6996 (21)	16107 ± 6220 (18)	21121 ± 6743 (12)
Significance†	NS	+	NS	NS	NS
Ratio $\frac{FM_x}{FC_x}$	1.06	0.70	0.78	0.85	0.77

* F = paternal cells responding M_x = maternal, C_x = control cells stimulating

§ The mean ± SD values have been calculated from the increment (FM_x–FF_x, FC_x–FF_x) cpm values of cultures harvested on day six.

† NS = not significant + = almost significant, $p < 0.05$ (Student's t test)

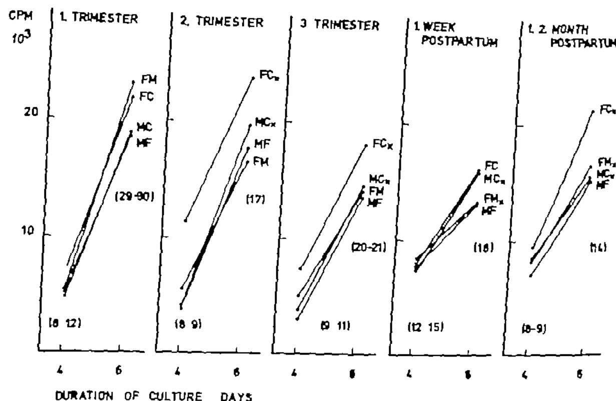


Fig 1 Time-course kinetics of MLC reactions during pregnancy and puerperium. M = maternal, F = paternal cells responding M_x F_x C_x = maternal paternal and control cells stimulating () = number MLC combinations studied.

Time-course of Maternal MLC Response

Time-course curves of maternal MLC responses were usually similar to those of other responses and the responses of maternal cells to paternal and control cells usually had similar course (Fig 1 for clarity the responses of control cells have been omitted) In the first postpartum week, however the reactions of maternal and paternal cells with each other were lower on day six than the other reactions.

of maternal serum is excluded (*Ceppellini et al* 1971 *Jones & Curzen* 1974 *Herva* 1976) The newborn baby has only the paternal transplantation antigens the mother has recently been in contact with, and to which she has perhaps been immunized. Therefore the possible immunological consequences of feto-maternal symbiosis are probably more readily reflected in the reactions between maternal and neonatal cells than in those between maternal and paternal cells, especially when primiparous pregnancies are studied.

The weaknesses of MLC reaction as a test for presensitization to transplantation antigens must also be remembered. Usually the accelerated secondary MLC response after in vivo sensitization lasts only a few weeks and cannot be induced again by booster immunizations (*Bondevik & Thorsby* 1974) MLC reaction or its inhibition by recipient serum have not proven useful in clinical rejection diagnostics (*Stiller et al.* 1976) or in demonstrating specific tolerance to donor cells (*Debray-Sachs et al.* 1973) even though MLC inhibition may be a sensitive test for allo antibodies (*Suciu-Foca et al.* 1974)

The effect of possible cytotoxic maternal lymphocytes (*Timonen & Saksela* 1976) on the MLC reactions between maternal and paternal (or neonatal) cells should be clarified. Cytotoxic cells could suppress MLC reactions by eliminating stimulating cells this would also explain the slight suppression in MLC reactions between maternal and paternal cells after delivery observed in this work

In conclusion, no apparent specific alterations were found in maternal MLC reactivity against fetal transplantation antigens as presented by paternal lymphocytes in any phase between the first trimester of pregnancy and late puerperium. The possible immunological consequences of feto-maternal symbiosis were thus not reflected in MLC this may be due either to the weaknesses of the present experimental approach in detecting presensitization, or to the absence of specific immunological modifications as a physiological buffering mechanism in pregnancy MLC blocking antibodies were not regularly produced during pregnancy Nonspecifically suppressed reactivity of maternal lymphocytes and the in vitro immunosuppressive effect of maternal serum may have a role in the non-rejection of the fetus.

Our sincere thanks are due to Mrs *Anni Jokinen* *Anne-Liisa Lindroos* *Anne-Liisa Hiltunen* *Leppa Hongisto* and *Terttu Peltola* for their invaluable help in collecting the specimens. We thank Mrs *Marja Sistonen* for excellent technical assistance Mrs *Ritva Rytö* M.Sc., for her help in processing the results, and Miss *Raili Lindh* for artistic and secretarial performance.

This study was supported by the *Sigrid Juselius Foundation* and *President J. K. Paasikivi Foundation.*

REFERENCES

Ahrons S HL-A antibodies Influence on the human foetus. Tissue Antigens *1* 129–136 1971

Amos D B., Bashir H Boyle W., MacQueen M & Tukkainen A A simple microcytotoxicity test. Transplantation *7* 220–222, 1969

Anderson J M Transplantation—nature's success. Lancet *ii* 1077–1082, 1971

Bach F H & Hirschhorn, K. Lymphocyte interaction A potential histocompatibility test in vitro Science *142* 813–814 1964

Bain B., Vas M R. & Lowenstein L. The development of large immature mononuclear cells in mixed leukocyte cultures. Blood *23* 108–116 1964

Beer A E. & Billingham, R. E. Immunobiology of mammalian reproduction. Adv Immunol. *14* 1–84 1971

Billington W D Immunological aspects of normal and abnormal pregnancy Eur J Obstet. Gynec. Reprod. Biol. *5* 147 154 1975.

Bondevik H Helgesen A Thorsen, A. B & Thorsby E.: A mixed lymphocyte culture microtechnique and its application in different family and unrelated combinations. Tissue Antigens *4* 469–481 1974

Bondevik H & Thorsby E. The human mixed lymphocyte culture (MLC) interaction after in vivo alloimmunization. I Kinetics. Cell. Immunol. *11* 409–418, 1974

Bonnard G D & Lemos L. The cellular immunity of mother versus child at delivery: Sensitization in unidirectional mixed lymphocyte culture and subsequent ^{51}Cr-release cytotoxicity test. Transplant. Proc. *4* 177–180, 1972.

In addition to the last mentioned mother one of the mothers developed lasting antibodies to paternal cells during the study In this case, antibodies were detected in the third trimester and at delivery Unfortunately the MLC inhibiting effect of this mother's serum was not tested postpartum. In the third trimester, the serum inhibited MLC reactions to 0.30–0 60 of control values.

Thus, three of the four mothers' sera having MLC inhibiting effect postpartum were cytotoxic at some time during pregnancy or puerperium. In addition one MLC inhibiting postpartum serum was not tested for cytotoxicity and one cytotoxic serum was not tested for MLC inhibition in postpartum period.

DISCUSSION

From the results presented we conclude that MLC reactivity of maternal cells to paternal cells is weakened only slightly if at all, in pregnancy Other workers have found a significant difference between maternal MLC responses to paternal and to unrelated cells (*Ceppellini et al.* 1971 *Lewis et al* 1966, *Jenkins & Hancock* 1972) the difference being more pronounced in multiparous than in primiparous women (*Lewis et al* 1966 *Jenkins & Hancock* 1972) In one of these studies, the inhibitory effect of maternal serum was shown to be responsible for the difference (*Jenkins & Hancock* 1972) in another this effect was not experimentally excluded (*Lewis et al* 1966) In the present work only primi or secundiparous women were studied the effect of parity on MLC will be analyzed in a later study which includes multiparous (six or more pregnancies) women.

To be able to express MLC reaction results reliably as relative responses and thus to detect possible slight modifications in maternal MLC reactivity during pregnancy selected control cells or a control cell pool should be used as a reference cell population (see *Thorsby et al* 1974) Otherwise, the wide natural variation of MLC reactions between in this respect unselected individuals and the considerable technical variation between the results of different experiments tend to mask the possible differences and alterations.

In agreement with earlier observations (*Ceppellini et al* 1971 *Jones & Curzen* 1973) we found MLC reactions of pregnant women's cells to be nonspecifically weaker than the reactions of other cells in our work this finding was compromized by differences in the treatment of cells before culturing

Cultures were harvested on days four and six In culture conditions very similar to our (*Bondevik & Thorsby* 1974) peak MLC response of cells from alloimmunized donors occurs on day four Time-course kinetics of maternal MLC response was apparently not influenced by the pregnancy This agrees with the findings from MLC experiments between maternal and neonatal cells (*Ce... et al.* 1974 *Herva* 1976)

MLC suppression by maternal serum strongest at delivery was demonstrated in this work in agreement with earlier observations (*Kasakura* 1971 *Leikin* 1972 *Jones et al* 1973) The inhibitory effect of maternal serum after delivery possibly caused by antibodies, was strong in three (12 per cent) and weak in two (8 per cent) of the mothers studied postpartum (total 5/23 22 per cent) Cytotoxic antibodies were found in 2 (9 per cent) of the mothers studied postpartum. These observations are similar to those reported by other workers (*Robert... et al* 1973 *Ahrons* 1971) According to ... recent studies (*Doughty & Gelsthorpe* 197... *I ves et al* 1976) and our results, cytotoxic antibodies detectable during pregnancy may in some cases disappear later in pregnancy. In our case, a relation seems to exist between cytotoxic antibodies and later MLC inhibition.

Several studies have shown that MLC responses of mother's cells to the cells of her newborn baby are quite often weak or negative (*Ceppellini et al* 1971 *Jenkins & Good* 1972 *Jones & Curzen* 1974 *Lawler et al* 1975 *Herva* 1976) even when the effect

Acta path. microbiol. scand. Sect. C, 85: 107–114, 1977

PSEUDOMONAS AERUGINOSA INFECTION IN CYSTIC FIBROSIS

Bactericidal Effect of Serum from Normal Individuals and Patients with Cystic Fibrosis on P. aeruginosa *strains from Patients with Cystic Fibrosis or other Diseases*

NIELS HØIBY* and SANTE OLLING**

*Statens Seruminstitut, Department of Clinical Microbiology at Københavns Kommunes Hospital i Hvidovre, and the Paediatric Clinic TG, Rigshospitalet, Copenhagen, Denmark
**Department of Immunology, Institute of Medical Microbiology and Institute of Pathology II, University of Göteborg, Göteborg, Sweden

Høiby, N. & Olling, S. *Pseudomonas aeruginosa* infection in cystic fibrosis. Bactericidal effect of serum from normal individuals and patients with cystic fibrosis on *P. aeruginosa* strains from patients with cystic fibrosis or other diseases. Acta path. microbiol. scand. Sect. C, 85: 107–114, 1977.

P. aeruginosa strains originating from the respiratory tract of patients with cystic fibrosis (CF) and patients with other diseases (non-CF) were analysed with regard to their sensitivity to the bactericidal activity of human serum. *P. aeruginosa* strains isolated from CF patients were more sensitive than strains from non-CF patients to the bactericidal activity of normal human serum. The bactericidal activity was heatlabile. As regards the sensitivity to normal human serum, mucoid and non-mucoid variants were not found to differ. Strains originating from chronically infected CF patients with many precipitins against these bacteria did not differ with respect to serum sensitivity from strains originating from intermittently colonized CF patients without *P. aeruginosa* precipitins. Compared with normal sera, CF sera showed similar or higher bactericidal activity against a panel of *P. aeruginosa* strains. In this respect, any difference between CF sera with precipitins and CF sera without precipitins against *P. aeruginosa* was not found. Sera from three CF patients chronically infected with *P. aeruginosa*, and with many precipitins against these bacteria, showed a selective inability in bactericidal activity against the patients' own *P. aeruginosa* isolate possibly reflecting the presence of "bactericidal blocking" antibodies.

Key words: *P. aeruginosa*; bactericidal serum; cystic fibrosis; "bactericidal blocking" antibodies.

S. Olling, Department of Immunology, Institute of Medical Microbiology, Guldhedsgatan 10, S-413 46 Göteborg, Sweden.

Received 13.viii.76 Accepted 25.x.76

Chronic pulmonary infections with mucoid strains of *Pseudomonas aeruginosa* are common in patients with cystic fibrosis (CF); these infections are associated with a pronounced antibody response (11, 17, 18, 29). However, elimination of these bacteria from the respiratory tract is not effected by the immune response (11, 17).

The majority of *P. aeruginosa* strains iso-

Bøyum A Isolation of mononuclear cells and granulocytes from human blood. Scand. J clin Lab Invest. *21* suppl *97* 77–89 1968.

Carr M C Stites D P & Fudenberg H H Cellular immune aspects of the human fetal maternal relationship III Mixed lymphocyte reactivity between related maternal and cord blood lymphocytes. Cell Immunol *11* 332–341 1974

Ceppellini R Bonnard G D Coppo F Miggiano V C & Pospisil M Mixed leukocyte culture and HL-A antigens I Reactivity of young fetuses newborns and mothers at delivery Tranplant. Proc. *3* 58–63 1971

Debray-Sachs M Dimitriu A Bach M A & Hamburger J Mixed lymphocyte culture in human renal allograft recipients Cell. Immunol. *7* 181–183 1973

Doughty R W & Gelsthorpe K Some parameters of lymphocyte antibody activity through pregnancy and further eluates of placental material Tissue Antigens *8* 43–48, 1976

Hartemann R J Segall M Bach M L & Bach F H Histocompatibility matching. IV Miniaturization of the mixed leukocyte culture test A preliminary report. Transplantation *11* 268–273 1971

Herva E. Mixed lymphocyte culture reactivity of neonatal lymphocytes. Lancet *i* 919–920 1976

Herva E. Comparison of microtitre plates with flat bottomed and round-bottomed wells for mixed lymphocyte culture (MLC) Acta path. microbiol scand Sect. C *85* 90–98, 1977

Häyry P & Defendi V Mixed lymphocyte cultures produce effector cells An in vitro model for allograft rejection Science *168* 133–135 1970

Jenkins D M & Good S Mixed lymphocyte reaction and placentation Nature New Biol *240* 211–212 1972

Jenkins D M & Hancock K W Maternal unresponsiveness to paternal histocompatibility antigens in human pregnancy Transplantation *13* 618–619 1972

Jones E & Curzen P The immunological reactivity of maternal lymphocytes in pregnancy J Obstet Gynaecol Br Commonw *80* 608–610 1973

Jones E & Curzen P Blocking one-way maternal foetal MLR Nature *250* 791 1974

Kasakura S A factor in maternal plasma during pregnancy that suppresses the reactivity of mixed leucocyte cultures. J Immunol. *107* 1296–1301 1971

Lancet The fetus as a homograft. Lancet *ii*. 335–336, 1973

Lawler S D Ukaejiofo E O & Reeves B R Interaction of maternal and neonatal cells in mixed-lymphocyte cultures. Lancet *ii*. 1185–1187 1975

Leikin S Depressed maternal lymphocyte response to phytohemagglutinin in pregnancy Lancet *ii* 43 1972.

Leventhal B G Buell D N Yankee R Rogentine G N Jr & Terasaki P. The mixed leucocyte response effect of maternal plasma. In *Harris J E* (Ed.) Proc. Fifth Leucocyte Culture Conference. Academic Press, New York 1970 p 473–486

Lewis J Jr Whang J., Nagel B., Oppenheim, J J & Perry S Lymphocyte transformation in mixed leukocyte culture in women with normal pregnancy or tumors of placental origin. Am. J Obstet. Gynec. *96* 287–290, 1966.

Robert M Betuel H & Revillard J P. Inhibition of the mixed lymphocyte reaction by sera from multipara. Tissue Antigens 3 59–56, 1973

Stiller C R Sinclair N R StC Abrahams S McGirr D Singh T Howson W T & Ulan R A Anti-donor immune responses in prediction of transplant rejection. New Engl. J Med. *294* 978–982, 1976

Suciu-Foca N Buda J A Thiem T & Reemtsma K Inhibition of mixed leukocyte culture reactivity by sera from hemodialysed patients and transplant recipients as an indicator of isoimmunization. Lab. Invest. *31* 1 3 1974

Sorensen F S The mixed lymphocyt culture interaction. Techniques and immunogenetics. Acta path. microbiol scand. Sect. B. Suppl 230 1972.

Thorsby E. The human major histocompatibility system. Transplant. Rev *18* 51–129 1974

Thorsby E. du Bois R. Bondevik H., Dupont B Eijsvoogel V., Hansen J A Jersild C., Jorgensen F Kissmeyer Nielsen F Lamm L U Schellekens P Th. A Svejgaard A & Thomsen M Joint report from a mixed lymphocyte culture workshop Tissue Antigens *4* 507 525 1974

Timonen T & Saksela F Istukan ja sikiön immunologinen sopeutuvuus. In *Kaitinen-Jääskeläinen M Wartiovaara J & Saxen L* (Eds.) Lääketieteellinen kehitysbiologia. Duodecim, Helsinki 1976 p. 285–296.

Tiwari J Gelabert A & Castillo R HLA antibodies and period of gestation Decline in frequency of positive sera d ring last trimester Tissue Antigens *7* 209–212 1976.

TABLE 1 *The Sensitivity of the Bacteria of Panel of 5 P aeruginosa Strains and 1 E. coli Strain to the Bactericidal Activity of Sera from 33 Normal Persons (30 Adults and 3 Children)*

	P aeruginosa					*E. coli*
	81	82	231	304	345	924 × 2831
Res	1	30	1	3	1	
Im	6	3	4	5	5	
Sens	26		28	25	27	33
Total	33	33	33	33	33	33

(Res = resistant, Im = intermediate Sens = very sensitive to the bactericidal activity of normal human serum The strains are indicated by their collection numbers)

broth was added to each tube. The anticomplementary activity of the BHI broth (25 *Olling* unpublished observations) and the dilution of the system inhibit further killing. All tubes were again incubated until growth was observed which usually took 8–10 h. The tubes were closed, the contents were mixed several times and the optical density measured on a spectrophotometer The results were divided into 3 classes by comparison with the 2 scale tubes resistant (Res) = less than 50 per cent of the bacteria had been killed intermediately sensitive (Im) = 50 per cent 99 per cent of the bacteria had been killed and very sensitive (Sens) = more than 99 per cent of the bacteria had been killed Controls were included to ensure sterility of the ingredients used.

Statistical Methods

The Chi-square test was employed. Level of statistical significance 5 per cent (double-tailed tests) (10)

RESULTS

The sera from the 30 blood donors and the 3 normal children gave basically similar patterns of killing characteristic of each of the strains in the panel of 5 *P aeruginosa* strains and one *E coli* strain (Table 1)

No significant reduction in bacterial growth was obtained if heat-inactivated sera (see Patients and Methods) from normal persons or from patients were used.

The sensitivity of mucoid and non-mucoid variants of *P aeruginosa* to the bactericidal activity of normal serum was not found to differ significantly (Table 2)

The sensitivity of *P aeruginosa* strains from CF patients to the bactericidal effect of normal serum was significantly higher than that of strains from the respiratory tract of non-CF patients ($P < 0.005$) (Table 3)

The sensitivity to the bactericidal activity of normal serum in strains from chronically infected CF patients with *P aeruginosa* precipitins and strains from CF patients intermittently colonized with *P aeruginosa* without precipitins against these bacteria was not found to differ significantly (Table 4)

Sera from CF patients were then examined for bactericidal activity against the panel of 5 *P aeruginosa* strains and one *E. coli* strain. Any significant differences between the bactericidal activity of sera from CF patients without precipitins against *P aeruginosa* (Table 5) and sera from CF patients with many precipitins against these bacteria were not found (Table 6) One of the *P aeruginosa* strains (number 82) however was significantly more sensitive to the bactericidal activity of CF sera than to normal sera ($p < 0.0005$) (Tables 1 5 & 6)

The serum bactericidal sensitivity of *P aeruginosa* strains from CF patients with many precipitins against these bacteria were examined using the patients' own serum vs. normal serum (Table 7) In 16 cases, the homologous strains gave similar results in the bactericidal sensitivity tests using normal sera and patients' sera. In 5 cases, however the strains were resistant when the patient's own serum was used but sensitive if sera from 7 different normal subjects were used. These 5 sera came from 3 CF patients, one

lated from non-CF patients appear to be resistant against the bactericidal activity of normal and convalescent sera (9 35 37) Moreover chronic infections of the lung and urinary tract in non-CF patients have been associated with the development of "bactericidal blocking" antibodies of the IgG class (15 31, 34 35) Similar blocking antibodies of the IgA class in patients suffering from meningococcal infections have been described (14) In contrast, persistent asymptomatic urinary tract infection caused by *Escherichia coli* may be associated with a successive increasing sensitivity of the infecting organism to the bactericidal activity of normal human serum (22)

The aim of the present study was to investigate and compare the serum sensitivity of *P aeruginosa* strains isolated from the respiratory tract of CF patients and from patients suffering from other diseases. It was also attempted to determine whether any differences between the sensitivity of concomitantly isolated mucoid and non-mucoid variants of *P aeruginosa* from CF patients could be found (1) In addition, the bactericidal effect of patients sera against their own infecting strains was compared with the effect against a panel of other strains in order to detect any selective inability in serum bactericidal activity

PATIENTS AND METHODS

Patients and Bacterial Strains

P aeruginosa were isolated and identified from the sputum or secretion obtained by endolaryngeal suction from CF patients and patients with other diseases (non-CF patients) as previously described (17 20) The *P aeruginosa* strains from CF patients originated from 41 of the patients treated in the CF Clinic TG Rigshospitalet Copenhagen (17) Twenty four of these CF patients suffered from chronic pulmonary infection with *P aeruginosa* (17) and all these patients produced multiple precipitins against *P aeruginosa* as revealed by crossed immunoelectrophoresis (18) The remaining 17 CF patients were intermittently harbouring *P aeruginosa* in the lower respiratory tract (17) and did not produce detectable precipitins against *P aeruginosa* (18) In total 115 *P aeruginosa* isolates from the CF patients were investigated, including mucoid as well as non mucoid isolates according to previously published criteria (1). Seventy-one previously described strains of *P aeruginosa* isolated from the respiratory tract of 71 hospitalized non-CF patients suffering from chronic bronchitis, bronchiectasia or tracheostomized patients were used as control strains (20) Only one of the 71 strains was mucoid. Twenty-five of these originated from patients with precipitating antibodies against *P aeruginosa* as revealed by crossed immunoelectrophoresis (18) 23 strains came from patients without such antibodies and 23 strains were from patients whose sera had not been investigated for *P aeruginosa* antibodies.

The bacteria were isolated during 1973 and stored as reported previously (1 20) they were mailed in agar slants to Göteborg for the serum bactericidal sensitivity test. All strains were subcultured before mailing to check the mucoid or non-mucoid colony morphology

Five of the CF strains and a serum sensitive rough *E. coli* strain were used as a panel in some of the investigations.

Serum

Blood was obtained by venipuncture allowed to clot for 1 h and serum stored at –80° C for 1 3 months until mailed by air at 70 C to Göteborg For the homologous serum bactericidal sensitivity test, 24 pairs of sera and *P aeruginosa* isolates originating from 17 CF patients were obtained. The serum and *P aeruginosa* strain from each of the patients were obtained on the same day These sera all contained multiple (6–50) precipitating antibodies againstt *P aeruginosa* (18) Sera from 22 CF patients without *P aeruginosa* infection and without detectable precipitins against these bacteria, and sera from 30 normal healthy adults and 5 normal, healthy children were used as controls. None of the patients received antibiotics at the time of venipuncture.

Serum Bactericidal Sensitivity (SBS) Test

SBS was measured as described earlier (26) Brain Heart Infusion (BHI) (Difco Laboratories, Detroit, Mich., U S.A.) broth cultures were grown overnight at 37 C The cultures were diluted 1 100 in Hanks solution and 0 1 ml was added to tubes each of which contained 0 1 ml fresh or heat inactivated (56 C for 10 min) serum either from the patient or from a normal person. In addition two tubes containing Hanks solution instead of serum were inoculated with 0 1 ml of the same culture but diluted 1 200 and 1 10 000 These tubes were used as a "scale" for comparison and thus contained 50 per cent vs. 1 per cent of the number of bacteria in the tubes with serum samples. After incubation at 37 C for 30 min, during which time the serum sensitive bacteria are killed, 2 ml of the

TABLE 6. *The Sensitivity of the Bacteria in the Panel of 5 P aeruginosa Strains and 1 E. coli Strain to the Bactericidal Activity 1 Sera from 18 Cystic Fibrosis Patients with Pr cipitins against P aeruginosa*

	P aerugin sa					*E. coli*
	81	82	231	304	345	924 × 2831
Res		2			1	
Im	1	10			4	
Sens	17	1	13	12	3	18
Total	18	13	13	12	8	18

(Res = resistant, Im = intermediate, Sens = ery sensitiv to the bactericidal activity of cystic fibrosis serum The strains are indicated by their collection numbers)

TABLE 7 *The Sensitivity of 21 P aeruginosa Isolates to the Bactericidal Activity of Patients own Serum vs. Normal Serum. Twenty-one Pairs of P aeruginosa Isolate and Homologous Sera from 16 Patients with Cystic Fibrosis and many P ecipitins against These Bacteria*

		Patient s serum			
		Res	Im	Sens	Total
Normal serum	Res	1			1 (5 %)
	Im	1	3	1	5 (24 %)
	Sens	5		10	15 (71 %)
	Total	7 (33 %)	3 (14 %)	11 (53 %)	21

(Res = resistant, Im = intermediate, Sens = very sensitive to the bactericidal activity of homologous cystic fibrosis serum s. normal serum. I 3 cases the *P aeruginosa* strains grew too slowly to be tested and these 3 cases are omitted)

of whom has later succumbed. Sera and *P aeruginosa* strains were obtained twice from these 3 patients, the interval between the two samplings being 5–7 months. At the first sampling, 2 of the 3 strains were resistant to the patients sera at the second sampling all 3 strains were resistant to the homologous sera. All 5 sera were bactericidal towards the panel of the other *P aeruginosa* strains and the *E. coli* strain

DISCUSSION

Normal human serum is bactericidal to many strains of different Gram-negative bacterial species previous studies have indicated that resistance to the bactericidal activity in serum may be a factor which contributes to the virulence of these organisms (14 19 22, 26 30 34 35 37) The bacterial factors as well as serum factors involved in the bactericidal activity are not completely identified. Smoothness, amount of endotoxin and of K antigen in the organism have been shown to be related to resistance against the bactericidal effect (16, 28, 32) Serum components involved in the bactericidal activity include the classical way of complement activation through antibodies and C1–C9 as well as the alternative pathway for complement activation including properdin (12, 27 33) In addition, *Mo sen & Skarnes* have recently suggested a third mode of bacterial killing via complement activation (24)

In the present study the sensitivity to the bactericidal activity of normal human sera

TABLE 2. *The Sensitivity to the Bactericidal Activity of Normal Human Serum of 31 Pairs of Mucoid vs non Mucoid Variants of* P aeruginosa *from the Respiratory Tract of 19 Patients with Cystic Fibrosis*

		Mucoid variant			Total
		Res	Im	Sens	
Non mucoid variant	Res	2		1	3 (10 %)
	Im	1	5	3	9 (29 %)
	Sens	1	1	17	19 (61 %)
	Total	4 (13 %)	6 (19 %)	21 (68 %)	31

(Res = resistant, Im = intermediate, Sens = very sensitive to the bactericidal activity of normal human serum)

TABLE 3 *The Sensitivity to the Bactericidal Activity of Normal Human Serum of* P aeruginosa *Strains from 41 Patients with Cystic Fibrosis vs 71 non-Cystic Fibrosis Patients*

	Res	Im	Sens	Total
Cystic fibrosis	9 (22 %)	7 (17 %)	25 (61 %)	41
Non-cystic fibrosis	39 (55 %)	20 (28 %)	12 (17 %)	71

(Res = resistant, Im = intermediate, Sens = very sensitive to the bactericidal activity of normal human serum)

TABLE 4 *The Sensitivity to the Bactericidal Activity of Normal Human Serum of* P aeruginosa *Strains from 24 Patients with Cystic Fibrosis with vs 17 Cystic Fibrosis Patients without Precipitins against* P aeruginosa

	Res	Im	Sens	Total
Cystic fibrosis with precipitins	5 (21 %)	5 (21 %)	14 (58 %)	24
Cystic fibrosis without precipitins	5 (29 %)	3 (18 %)	9 (53 %)	17

(Res = resistant, Im = intermediate Sens = very sensitive to the bactericidal activity of normal human serum)

TABLE 5 *The Sensitivity of the Bacteria in the Panel of 5* P aeruginosa *Strains and 1* E. coli *Strain to the Bactericidal Activity of Sera from 22 Cystic Fibrosis Patients* without *Precipitins against* P aeruginosa

	P aeruginosa					*E. coli*
	81	82	231	304	345	924 × 2831
Res						
Im	2	17			7	
Sens	20	2	14	12	4	22
Total	22	19	14	12	11	22

(Res = resistant, Im = intermediate Sens = very sensitive to the bactericidal activity of cystic fibrosis serum. The strains are indicated by their collection numbers)

of antigenic "drift" or "conversion" occuring as a result of an adjustment to the host's immune response (22)

This work was supported by grants from the *Thorald Madsen Legat* the *Danish Medical Research Council* (N.H.) the *Medical Faculty University of Göteborg*, and the *Swedish Medical Research Council* (project no. 215) (S.O.) Mrs. *Annal Bathien Inga Engberg* and *Liselotte Johansson* are thanked for skilful technical assistance.

REFERENCES

1. *Bergan, T & Høiby N* Epidemiological markers for *Pseudomonas aeruginosa*. 6. Relationship between concomitant non-mucoid and mucoid strains from the respiratory tract in cystic fibrosis. Acta path. microbiol. scand. Sect. B 83 553–560 1975
2. *Biggar W D Holmes B. & Good R. A* Opsonic defect in patients with cystic fibrosis of the pancreas. Proc. Nat. Acad. Sci. U.S.A 68 1716–1719 1971
3. *Bjornson A. B. & Michael, J G* Biological activities of rabbit immunoglobulin M and immunoglobulin G antibodies to *Pseudomonas aeruginosa* Infect. and Immun. 2 453–461 1970.
4. *Bjornson A B & Michael J G* Contribution of humoral and cellular factors to the resistance to experimental infection by *Pseudomonas aeruginosa* in mice. I Interaction between immunoglobulins, heat-labile serum factors, and phagocytic cells in the killing of bacteria. Infect and Immun. 4 462–467 1971
5. *Bjornson A. B & Michael, J G* Contribution of humoral and cellular factors to the resistance to experimental infection by *Pseudomonas aeruginosa* in mice II Opsonic, agglutinative, and protective capacities of immunoglobulin G anti-Pseudomonas antibodies. Infect. and Immun 5 775–782, 1972
6. *Bjornson A B & Michael J G* Factors in human serum promoting phagocytosis of *Pseudomonas aeruginosa*. I Interaction of opsonins with the bacterium J Infect. Dis. 130 Suppl 119–125 1974
7. *Bjornson A. B. & Michael, J G.* Factors in human serum promoting phagocytosis of *Pseudomonas aeruginosa* II Interaction of opsonins with the phagocytic cell. J Infect. Dis 130 Suppl 127–131 1974.
8. *Boxerbaum B K gumba, M & Matthews L* Selective inhibition of rabbit alveolar macrophages by cystic fibrosis serum. Am. Rev Resp Dis 108 777 783 1973
9. *Crowder J G Dech H B., Fisher M W & White A.*: Heatstable opsonins in sera of patients with Pseudomonas infections. J Lab. clin. Med. 83 833–839 1974
10. *Diem K* (Ed.): Documenta Geigy Scientific Tables. J R. Geigy S A., Basel 1962, p. 36.
11. *Doggett R. G & Harrison G M*: Significance of the bacterial flora associated with chronic pulmonary disease in cystic fibrosis. 5th International cystic fibrosis conference, Churchill college Cambridge Pp. 175–188 1969
12. *Goldman J N & Austen K. F.* Reaction mechanisms of Nascent C_{56} (reactive lysis) II Killing of a rough form of *Escherichia coli* by C_{56-7}, C8 and C9 Infect. Dis. 129 444 450 1974
13. *Govan J R. W.*: Mucoid strains of *Pseudomonas aeruginosa* the influence of culture medium on the stability of mucus production. J Med. Microbiol 8 513–522, 1975
14. *Griffiss J M.*: Bactericidal activity of meningococcal antisera Blocking by IgA of lytic antibody in human convalescent sera. J Immunol. 114 1779–1784 1975
15. *Guttman R M & Waisbren B A.*, Bacterial blocking activity of specific IgG in chronic *Pseudomonas aeruginosa* infection. Clin. exp. Immunol. 19 121–130, 1975
16. *Howard C J & Glynn A A* The virulence for mice of strains of *Escherichia coli* related to the effect of K antigens on their resistance to phagocytosis and killing by complement. Immunology 20 767–777 1971
17. *Høiby N.*: Epidemiological investigations of the respiratory tract bacteriology in patients with cystic fibrosis. Acta path. microbiol. scand. Sect. B, 82 541 550, 1974
18. *Høiby N* *Pseudomonas aeruginosa* infection in cystic fibrosis. Relationship between mucoid strains of *Pseudomonas aeruginosa* and the humoral immune response. Acta path. microbiol. scand. Sect. B, 82 551 558, 1974
19. *Høiby N* Normally occurring precipitating antibodies against *Pseudomonas aeruginosa*. Prevalence, specificities and titres. Scand. J Immunol. 4 Suppl. 2, 197–202, 1975
20. *Høiby N* Prevalence of mucoid strains of *Pseudomonas aeruginosa* in bacteriological specimens from patients with cystic fibrosis and patients with other diseases. Acta path. microbiol. scand. Sect. B, 83 549–552, 1975
21. *Koch C., Høiby N & Wulf A.* Nitroblue tetrazolium (NBT)-reduction by human peripheral neutrophil granulocytes in the presence of bacterial antigens. Evidence for immune complex ingestion. Acta path. microbiol. scand Sect. C, 83 144–156 1975.
22. *Lindberg, U., Hanson L. A Jodal, U Lidin*

was characteristic of each of the strains of *P aeruginosa* (Table 1) It is thus possible to generalize and call a *P aeruginosa* strain resistant, intermediate or very sensitive to the bactericidal activity of normal human serum in accordance with results obtained previously using *E coli* strains (26) In all cases, the bactericidal activity was dependent on heat labile factors whether the latter were complement factors was not further investigated.

It is surprising that the sensitivity of mucoid and non mucoid variants of *P aeruginosa* in the serum was not found to differ However the mucoid substance is not firmly bound to the bacterial cells, but diffuses into the environment this might in part explain why mucoid and non mucoid variants are of comparable sensitivity to the activity of serum in a fluid phase system (11) Moreover production of mucoid substance is dependent on a bacteriophage which is easily lost (23) Accordingly, mucoid *P aeruginosa* regularly split off non mucoid dissociants, and broth cultures appear to undergo dissociations more frequently than surface cultures (1 13 23 38) The results of the comparison of mucoid vs. non mucoid variants of *P aeruginosa* in the present fluid phase system might therefore be influenced by dissociation from mucoid to non mucoid dissociants during the test. This dissociation occurs also *in vivo* however and may play an important role in the host parasite relationship (1 11 13 17 18 23 29 38)

Most *P aeruginosa* strains isolated from clinical sources have been reported to be resistant to the bactericidal activity of normal and convalescent serum (9 35 37) In accordance with this, only few of the *P aeruginosa* strains from non-CF patients were very sensitive to human serum In contrast, most strains from CF patients were very sensitive to the bactericidal activity of normal serum and, in this respect, differences between strains isolated from chronically infected patients with many precipitins against *P aeruginosa* and strains isolated from intermittently colonized patients without detectable precipitins against these bacteria were not found CF sera were equal to or better than normal sera in their ability to kill the panel of 5 *P aeruginosa* strains and the *E. coli* strain. In this respect, however any differences between sera containing many precipitins against *P aeruginosa* and sera without any detectable precipitins against these bacteria were not revealed. On the contrary some of the patients sera were less efficient than normal sera in killing the homologous strain This selective defect is most likely due to "bactericidal blocking" antibodies of the IgG or IgA class as described by other authors (14 15 31 34 35)

The present work has not revealed any increased bactericidal activity against *P aeruginosa* of sera from CF patients with *P aeruginosa* precipitins compared with sera from CF patients without such precipitins. However the protective role of antibodies in infections due to *P aeruginosa* is probably to promote phagocytosis—in particular type-specific phagocytosis—of the bacteria by neutrophils or macrophages (3–7 9 35–37) CF sera notably from patients with many *P aeruginosa* precipitins seem to be able to promote phagocytosis of *P aeruginosa* by human neutrophils, whereas CF sera possibly are defective as a source of opsonins when rabbit alveolar macrophages are mixed with *P aeruginosa* cells (2 8 21) Whether the latter defect is related to the occurrence of blocking antibodies remains to be studied.

Apparently CF patients are mainly colonized with serum sensitive strains of *P aeruginosa*. This might explain why these patients seldom, if ever suffer from bacteraemic infections caused by *P aeruginosa* (11 17) A similar situation is found in persisting infection of the urinary tract with *E coli*. These infections are caused mainly by serum-sensitive strains, and in a number of cases, such strains have been found to become increasingly sensitive to normal serum during follow up of untreated patients (22, 26)

The appearance of these sensitive *P aeruginosa* and *E. coli* strains in subjects infected repeatedly or chronically may be an example

Acta path. microbiol. scand. Sect. C, 85: 115–122, 1977

AN INDIRECT HAEMAGGLUTINATION TEST FOR DEMONSTRATION OF GONOCOCCAL ANTIBODIES USING GONOCOCCAL PILI AS ANTIGEN

I. Methodology and Preliminary Results

KARIN REIMANN and INGA LIND

Neisseria Department, Statens Seruminstitut, Copenhagen, Denmark

Reimann, Karin & Lind, Inga. An indirect haemagglutination test for demonstration of gonococcal antibodies using gonococcal pili as antigen. I. Methodology and preliminary results. Acta path. microbiol. scand. Sect. C, 85: 115–122, 1977.

An indirect haemagglutination (IHA) test for detection of gonococcal antibodies in human sera was established using purified pili as antigen. The majority of sera (44/51) positive in the gonococcal complement fixation test, were also positive in the IHA test with titres within the range 320–10,000. The results obtained for the following three groups of sera indicate a comparatively high specificity of the IHA test: 1) 52 sera from children below 12 years of age were all negative; 2) 98 per cent (184/188) of sera from blood donors were negative; 3) only sera from three adults out of 21 patients with meningococcal infections gave a positive reaction.

Key words: Gonococcal antibodies; indirect haemagglutination; gonococcal pili.

Karin Reimann, Neisseria Department, Statens Seruminstitut, Amager Boulevard 80, DK-2300 Copenhagen S, Denmark.

Received 10.xi.76 Accepted 28.xii.76

The correlation between the presence of particular colony types of *Neisseria gonorrhoeae* and virulence for man was demonstrated by *Kellogg et al.* (11, 12). The colony types T1 and T2 shown to be virulent for man were observed to be infectious in the chimpanzee (2) and in the chick embryo animal model (4). The virulent colony types were investigated by *Jephcott et al.* (9) and *Swanson et al.* (27), who demonstrated pili on virulent T1 and T2 gonococci and the absence of pili on avirulent T3, T4 and T5 gonococci.

In 1975 *Buchanan et al.* (6) published the first and so far the only report on the occurrence of antibodies to gonococcal pili in human sera. Using a radioimmunoassay they studied the level of antibodies to gonococcal pili in patients with gonococcal infection and in control groups. The present study was initiated by the promising results obtained in asymptomatically infected females, in which group 89 per cent showed an antibody level higher than that of the control groups. However, for screening larger populations the radioimmunoassay is a too expensive and time-consuming procedure. In this report an

Janson G Lincoln K & Olling S Asymptomatic bacteriuria in schoolgirls. II Differences in *Escherichia coli* causing asymptomatic and symptomatic bacteriuria Acta Paediatr Scand *64* 432–436 1975

23 *Martin D R* Mucoid variation in *Pseudomonas aeruginosa* induced by the action of phage J med Microbiol. *6* 111–113 1973

24 *Moreau S C & Skarnes R C* Complement mediated bactericidal system Evidence for a new pathway of complement activation. Science *190* 278–280 1975

25 *Muschel L. H & Treffers H P* Quantitative studies on the bactericidal actions of serum and complement. J Immunol *76* 1–27 1956

26 *Olling, S Hanson L. A Holmgren J Jodal U Lincoln K & Lindberg U.* The bactericidal effect of normal human serum on *E coli* strains from normals and from patients with urinary tract infections. Infection *1* 24–28 1973

27 *Reed W P & Albright E. L.* Serum factors responsible for killing of Shigella. Immunology *26* 205–215 1974

28. *Rowley D* Antibacterial action of antibody and complement. *In Kass E. & Wolff S* (Eds) Bacterial Lipopolysaccharides. The University of Chicago Press, Chicago 1973 p 162–167

29 *Schiotz P O & Høiby N* Precipitating antibodies against *Pseudomonas aeruginosa* in sputum from patients with cystic fibrosis specificities and titres determined by means of crossed immunoelectrophoresis with intermediate gel Acta path. microbiol. scand. Sect. C *83* 469–475 1975

30 *Simberkoff M S Ricupero I & Rahal, J R. Jr* Host resistance to *Serratia marcescens* infection serum bactericidal activity and phagocytosis by normal blood leucocytes. J Lab. Clin. Med. *87* 207–217 1976.

31 *Taylor P W* Isolation and characterization of a serum antibactericidal factor Clinical Science *43* 705–708 1972.

32 *Taylor P W.* Genetical studies of serum resistance in *Escherichia coli*. J Gen. Microbiol *89* 57–66 1975

33 *Taub W H & Kleber I* Selective activation of classical and alternative pathways of human complement by "promptly serum-sensitive" and "delayed serum-sensitive" strains of *Serratia marcescens* Infect. and Immun. *13* 1343–1346 1976

34 *Waisbren B A & Brown I.* A factor in serum of patients with persisting infection that inhibits the bactericidal activity of normal serum against the organism that is causing the infection. J Immunol. 97 431–437 1966.

35 *Young L. S & Armstrong D* Human immunity to *Pseudomonas aeruginosa*. I In-vitro interaction of bacteria, polymorphonuclear leukocytes, and serum factors. J Infect. Dis. *126* 257–276 1972

36 *Young L. S.* Human immunity to *Pseudomonas aeruginosa*. II Relationship between heat-stable opsonins and type-specific lipopolysaccharides. J Infect. Dis. *126* 277–287 1972.

37 *Young, L. S* Role of antibody in infections due to *Pseudomonas aeruginosa*. *J Infect.* Dis. *130 Suppl.* 111–118, 1974

38. *Zierdt C H & Schmidt P J* Dissociation in *Pseudomonas aeruginosa*. J Bact. 87 1003–1010 1964

were then washed twice in 1 per cent NRS and resuspended in 1 per cent NRS as 1 per cent suspension. SBRC treated in the same way with PBS pH 6.4 without antigen were used as controls. Sensitized SBRC were freshly prepared on each day of experiment.

d) Performance of the test: Serum specimens were inactivated at 56 C for 30 minutes and absorbed with an equal olume of packed, formalin-treated SBRC. The absorption was performed at 1 20 dilution of the serum in PBS, pH 6 4 for 10 minutes at room temperature. After centrifugation the supernatants were used for titrations, which were performed in disposable plates (Microtiter® U-shaped wells) The two-fold serial dilutions were made in 1 per cent NRS the volume in each well being 50 μl 25 μl of a 1 per cent suspension of sensitized SBRC was added to each well The tw highest serum concentrations of each serum sample were also tested with 1 per cent non-sensitized SBRC. Titration of positive and negativ reference sera and examination of sensitized and non-sensitized SBRC against 1 per cent NRS were included in each experiment. The plates were carefully shaken and covered. They ere left overnight at room temperature before reading.

For haemagglutination inhibition studies, 25 μl of the substance to be tested for inhibitory effect was added to each well 1 hour before the SBRC 25 μl 1 per cent NRS was added to the controls.

) Reading The agglutination pattern was graded from ++++ to 0 The ++++ and +++ indicated strongly positive reactions in hich the layer of SBRC had rolled down from the edges of the well. A ++ reaction showed an even layer of SBRC covering the bottom of the ell Formation of big ring was recorded as + and ery small ring or spot as 0 The titre of given specimen was recorded as the reciprocal alue of the lowest serum concentration giving + reaction In this context values >20 were considered positiv

Gonococ al omplement fixation test (GCF) The antigen was prepared and the test performed as described previously by *Reyn* (24). The highest serum concentration tested was 1 12. The titre as read as the reciprocal alue of the lowest serum concentration giving 60 per cent haemolysis

Meningococ al compl ment fixation test (MCF) The procedure was analogous to that used for the GCF

2-merc ptoethanol treatment Equal olumes of serum and 0 2 M 2-mercaptoethanol in PBS pH 7 2 were mixed and incubated at 37 C for 45 minutes Controls were incubated with equal olumes of PBS, pH 7 2. All samples were tested immediately in the IHA test without further treatment

Human sera. The following groups of sera were examined in the IHA test (the number in brackets indicates the strain from which the pili antigen was made): A) Serum specimens received with request for examination in GCF and giving positive reaction (82409) B) Serum specimens received from 12 adults and 9 children with evidence of meningococcal infection (82409 and 35209) C) Sera from children <12 years of age with unrelated disease (received with a request for either MCF or determination of antibodies to *Mycoplasma pneumoniae*) (82409) D) Blood donors (35209)

RESULTS

Gonococcal pili antigens Figs. 1 and 2 are electron micrographs of the two preparations of purified pili used as antigens in the IHA test in the present study Both preparations contained large amounts of pili and were slightly contaminated with small blebs. Morphologically the pili of strain 82409 are similar to pilus *a* described by *Novotny et al.* (20) (Fig 2) Preparations of pili from strain 35209 contained pili similar to both pilus *a* and pilus *b* the latter being illustrated in Fig. 1 Further morphological differences between the two preparations could not be established by means of the method employed. In preliminary experiments medium without antibiotics had been used. No change in morphology of the pili was noticed after the introduction of the antibiotics. The yield obtained from the bacterial growth of 200 plates (Petri dishes, 9 cm diameter) was 1 ml purified pili preparation containing about 3 mg protein per ml. For both antigen preparations used the optimal concentration for sensitization of SBRC was 2-3 μg protein per ml. A single preparation of 82409 pili was examined for content of polysaccharides (Anthrone reaction) and this was found to be less than 10 μg/ml (lower limit of the method) at a protein concentration of 0.34 mg/ml. Sufficient material for further biochemical analysis has not been available so far

The IHA test The purified pili antigen was adsorbed onto SBRC after treatment of the cells with formalin and tannic acid, whereas it did not adsorb onto untreated

indirect haemagglutination test using purified gonococcal pili as antigen is described, and the preliminary results obtained by examination of sera from selected groups of individuals are presented. The results obtained by screening various groups of patients attending a dermato-venereological out patient clinic will be reported in a subsequent paper

MATERIAL AND METHODS

Bacterial strains. Two strains of *Neisseria (N.) gonorrhoeae* (SS 87409/1969 SS 35209/1975) were selected for the production of pili antigen due to abundant formation of pili when grown on the medium described below The strains were kept in liquid nitrogen. Selective transfer of colony type 2 was made every day using a stereomicroscope with oblique substage illumination

Medium The modified Kellogg medium (11) consisted of Difco GC base medium, 36 g/litre plus 1 per cent supplement The supplement was composed of co-carboxylase 0 02 g L-glutamine 10 g, glucose 400 g ferric nitrate 0 5 g dissolved in 1 litre of distilled water The following antibiotics were added polymyxin B sulphate 25 IU/ml, nystatin 25 IU/ml and vancomycin 2 μg/ml.

Buffers and diluents a) 0 01 M TRIS buffer pH 8.0 b) 0 01 M TRIS buffer pH 7 0 c) phosphate buffered saline, pH 6 4 (PBS pH 6 4) (4 675 g Na_2HPO_4 $2H_2O$ 6 63 g KH_2PO_4 4.5 g NaCl dissolved in 1 litre distilled water) d) phosphate buffered saline, pH 7 2 (PBS pH 7 2) (4 672 g Na_2HPO_4 $2H_2O$ 1 53 g KH_2PO_4, 6.75 g NaCl dissolved in 1 litre distilled water) e) saline (9 0 g NaCl dissolved in 1 litre distilled water) f) 1 per cent normal rabbit serum (1 per cent NRS) Normal rabbit serum was inactivated at 56 C for 30 minutes and absorbed in the same way as the serum specimens (see below) The absorbed NRS was then diluted to a 1 per cent solution in PBS pH 6 4

Preparation of gonococcal pili antigen The inoculated Kellogg plates were incubated for 18–22 hours at 36 C in a moist atmosphere containing about 5 per cent CO_2. The growth was harvested into 1 ml 0 01 M TRIS buffer pH 8 0 per plate The bacterial suspension was shaken on a Whirlimixer for 1 minute and then centrifuged for 15 minutes at 2,500 g The purification of the pili which are present in the supernatant, was performed according to the method described by *Buchanan et al.* (6) The pili preparation was finally suspended in 0 01 M TRIS pH 7 0 containing 0 01 M NaN_3 and stored at 4 C. The protein concentration of the stock suspension was about 2 mg per ml It should be carefully resuspended before use.

Characterisation of the pili preparation 1) The protein content was determined by the method of *Lowry et al.* (16) using bovine serum albumin as standard 2) the presence of polysaccharides (total hexoses) was determined by the Anthrone reaction (10 p 528-529) 3) the pili preparations were examined by electron microscopy by *Jens Blom* M.D., Department of Biophysics, Statens Seruminstitut. Grids were placed on drops of the pili preparations negative staining was performed using 1 per cent ammoniummolybdate, pH 7.5 (see Figs. 1 and 2)

Immunization of rabbits. Young rabbits (about 2,200 g) with a negative gonococcal complement fixation test were used for immunization. Each group consisted of four rabbits. A pili preparation containing 0 25 mg protein per ml was mixed with an equal volume of Freund's complete adjuvant. Each rabbit received 1 ml of the mixture subcutaneously A booster injection of the same mixture was given 3 weeks later Blood samples were drawn for the first time 2 weeks after the last injection. Rabbit antisera to formalin-killed whole cells of the pili-producing strains of *N gonorrhoeae* and to strains of *N meningitidis* were produced as described previously (15)

Indirect haemagglutination (IHA) test Blood from a sheep previously selected as a suitable donor for sheep blood red cells (SBRC) to be used in IHA tests was collected in an equal volume of Alsevers solution (10 p. 149) and stored for 5–10 days at 4 C before use.

a) Formalin treatment SBRC were washed 6 times in saline and then treated with 7.5 per cent neutralized formalin solution as described by *Weinbach* (28) The SBRC were finally resuspended in saline as a 10 per cent suspension. The suspension was stable at least 6 months at 4 C.

b) Tannic acid treatment The formalin-treated SBRC were washed once in saline and resuspended as a 2 per cent suspension in PBS pH 7.2. Tannic acid (Merck) was dissolved 1 40 000 in PBS pH 7 2 and mixed with an equal volume of the 2 per cent suspension of formalin-treated SBRC. The mixture was incubated in a 37 waterbath for 30 minutes. Then the SBRC were centrifuged down, washed twice with PBS pH 6 4 and resuspended as a 2 per cent suspension in the same buffer This suspension could be used up to 6 weeks when stored at 4 C

c) Sensitization For each preparation of pili antigen the optimal concentration for sensitization was determined by chess-board titrations of different dilutions of the antigen against dilutions of the homologous rabbit antiserum. A 2 per cent suspension of tanned, formalin-treated SBRC in PBS pH 6 4 was mixed with an equal volume of antigen diluted in PBS pH 6 4 and incubated 1 hour in a 37 C waterbath. The sensitized SBRC

TABLE 1 *Comparison of Results Obtained by GCF (Gonococcal Complement Fixation Test) and IHA (Indirect Haemagglutination Test Using Gonococcal Pili as Antigen) in Selected Groups of Individuals*

Group	Number	Number of positive reactions in GCF	IHA
Adults			
Request for GCF and positive GCF	51	51	44
Request for MCF and positive MCF	12	7	3
Blood donors	188	1	4
Children <12 years			
Request for MCF	22	4	0
Unrelated disease	30	0	0

SBRC. The titres obtained for positive sera were slightly higher after sensitization of SBRC at pH 6.4 than after sensitization at pH 7.2 and therefore the former pH was used throughout this study. Based on seven consecutive examinations, the standard error for the titre of the positive reference serum of the IHA test with 82409 pili antigen was 0.25 (log_{10} titre). Based on 20 consecutive examinations, the standard error for the titre of the positive reference serum of the IHA test with 35209 pili antigen was 0.24 (log_{10} titre). Six human sera presumed to contain gonococcal antibodies (GCF positive, *v.s.*) were tested in the IHA test with either of the pili antigens and the results were identical. On the other hand, the haemagglutination inhibition experiments carried out showed that pili from strain 35209 did not inhibit the reaction between SBRC coated with pili 82409 and rabbit antiserum to pili 82409 and *vice versa*.

Rabbit antisera. A pool of GCF-negative normal rabbit sera was negative in the IHA test. Rabbit antisera to pili and to whole cells of the pili-producing strains showed titres within the range 8,000–320,000 when tested against the homologous pili antigen and positive, but somewhat lower titres when tested against the other pili antigen. All of 15 rabbit antisera to strains of *N. meningitidis* (groups A, B and C) were negative. In routine GCF the rabbit antisera to pili preparations were negative, whereas rabbit antisera to whole cells of pili producing gonococci and to meningococci were positive.

Human sera. Selected groups of sera were examined in the IHA test: 1) presumed pos-

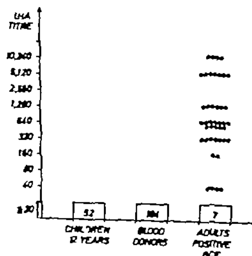


Fig. 3. Antibodies to gonococcal pili in human sera. IHA: indirect haemagglutination test using gonococcal pili as antigen. GCF: gonococcal complement fixation test. Results obtained by examination of presumed negative (children <12 years and blood donors) and presumed positive individuals (adults with positive GCF).

Figs. 1 and 2. 1) Purified pili of *N. gonorrhoeae* SS 35209/1975. 2) Purified pili of *N. gonorrhoeae* SS 82409/1969. Negative staining with 1 per cent ammoniummolybdate, pH 7.5. Magnification ×160,000. Arrows indicate small blebs, probably endotoxin.

1

0.1 µm

2

0.1 µm

We have chosen to evaluate another sensitive serological method requiring small amounts of antigen, the indirect haemagglutination (IHA) test. The test was established by means of purified pili from N. gonorrhoeae SS 82409/1969 (9) and rabbit antiserum to this pili antigen. The first approach to detect antibodies to gonococcal pili in human sera by means of the IHA test was the examination of a series of sera exhibiting positive GCF the majority of these (44/51) were positive in the IHA test (Fig. 3). the next step was a pilot study in which the immune response of eight patients with acute gonococcal infections was followed by several serological methods (unpublished data). Five out of these eight patients gave a positive reaction in the IHA test and two of these showed decrease in titre 3 months after treatment. The GCF was negative on all occasions. Thus the IHA test seemed to detect gonococcal antibodies in human sera.

A preliminary evaluation of the specificity of the IHA test was then established by examination of other groups of human sera 1) 52 sera from children <12 years of age were all negative 2) 184 out of 188 sera from blood donors (98 per cent) were negative 3) only sera from three adults among 21 patients with meningococcal infection gave a positive reaction. Rabbit antisera to N. meningitidis groups A, B and C were also negative. These facts indicate a comparatively high specificity of the IHA test.

In experiments using rabbit antisera to pili from various gonococcal strains (3 5 21) it has been shown that gonococcal pili are antigenically heterogeneous. On the other hand, the pili antigens used for demonstration of gonococcal antibodies in human sera both by *Buchanan et al.* (6) and in the present study were prepared from single strains. In the former study the proportion of positive findings was high, and in the present study the two different preparations gave identical results. This suggests that pili must also possess some antigens in common. Pili preparations are often contaminated with small blebs, probably free endotoxin, as thoroughly studied by *Novotny et al.* (20). Further studies on the antigenic structure of pili from different gonococcal strains and characterization of the pili preparations used are important for further development of the IHA test.

We thank *Jens Blom* Department of Biophysics, Statens Seruminstitut, for electron microscopic examination of the pili preparations and *Drs. Ibrandtsen* for skillful technical assistance.

The work was supported by grants from *Thorvald Madsen Fund* and the *World Health Organization*.

REFERENCES

1. *Bro-Jorgensen A. & Jensen T.* Gonococcal pharyngeal infections. Brit. J. Vener. Dis. *49* 491–499 1973
2. *Brown W. J. Lucas C. T. & Kuhn U. S. G.* Gonorrhoea in the chimpanzee. Brit. J. Vener. Dis. *48* 177–178, 1972.
3. *Buchanan T. M.* Antigenic heterogeneity of gonococcal pili. J. exp. Med. *141* 1470–1475 1975
4. *Buchanan T. M. & Gotschlich E. C.* Studies on gonococcus infection, III. Correlation of gonococcal colony morphology with infectivity for the chick embryo. J. exp. Med. *137* 196–200 1973
5. *Buchanan T. M. & Pearce W. A.* Pili as mediator of the attachment of gonococci to human erythrocytes. Infect. Immun. *13* 1483–1489 1976.
6. *Buchanan T. M. Swanson J. Holmes K. K., Kraus S. J. & Gotschlich, E. C.* Quantitative determination of antibody to gonococcal pili. Changes in antibody levels with gonococcal infection. J. clin. Invest. *52* 2896–2909 1973
7. *Danielsson D., Thyresson N., Falk V. & Barr J.* Serologic investigation of the immune response in various types of gonococcal infection. Acta derm.-venereol. (Stockh.) *52* 467–475 1972.
8. *Fletcher S., Miller R. & Nicol C. S.* Study of a passive haemagglutination test for gonorrhoea. Brit. J. Vener. Dis. *49* 508–510 1973.
9. *Jephcott A. E., Reyn A. & Birch-Andersen A.* Neisseria gonorrhoeae III. Demonstration of presumed appendages to cells from different colony types. Acta path. microbiol. scand. Sect. B, *79* 437–439 1971
10. *Kabat E. A. & Mayer M. M.:* Experimental Immunochemistry. 2. ed. Charles C. Thomas, Springfield, Illinois, USA, 1961

tive sera, 2) presumed negative sera and 3) sera presumed to contain antibodies to antigens shared by gonococci and meningococci induced during acute meningococcal infection.

The first group consisted of 51 sera representing various degrees of positive reactions in the GCF The majority of these (44/51) gave a positive reaction in the IHA test with titres within the range 320-10 000 (Table 1 Fig 3) Some of these sera were tested before and after treatment with 2 mercaptoethanol In all cases the positive reaction in the IHA test was due to mercaptoethanol resistant antibodies (IgG) 32 of these sera were examined in the MCF and 25 gave a positive reaction

The second group (presumed negative sera) originated from two materials (Table 1 Fig 3) 52 sera were from children <12 years of age, 9 of whom had meningococcal infection with positive MCF (and 4 concomitant positive GCF) These sera were all negative in the IHA test. The other material comprised 188 sera from blood donors. Four positive reactions were registered (Table 1 Fig 3) No information about previous gonococcal or other infections was available

The third group of sera were from 21 patients with evidence of meningococcal infection (Fig 4) Three sera gave a positive reaction in the IHA test These sera originated from the three oldest patients (36, 52 and 68 years of age) No information with regard to previous veneral disease was available The GCF was positive in 12/21 patients, including two of those mentioned above (IHA test positive) At the average the GCF showed a lower titre than the MCF (GCF $\log_{10}$ mean value 1 64 MCF $\log_{10}$ mean value 1 93)

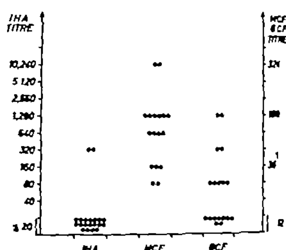


Fig 4 Comparison of MCF GCF and IHA titres in sera from 21 patients with evidence of meningococcal infection and positive MCF IHA indirect haemagglutination test using gonococcal pili as antigen. MCF meningococcal complement fixation test. GCF gonococcal complement fixation test.

DISCUSSION

The detection of the asymptomatic female carrier has remained the most important problem in the epidemiology of gonococcal disease (13 14 26) In most countries these patients are considered to be the reservoir which maintains a high incidence of the disease They are also the reservoir out of which the majority of cases of disseminated gonococcal infections arise.

The serological tests for gonorrhoea hitherto employed have been useful only in the diagnosis of complicated cases. In acute cases and asymptomatic patients the proportion of positive findings varies from a few per cent (1) to 18-40 per cent (7 23 26) As regards the gonococcal complement fixation (GCF) this variation can be referred mainly to differences in the level of antibodies registered as positive results. In addition antibodies to meningococci are known to give false positive results (22 25) The same lack of diagnostic specificity and sensitivity is the drawback also of more recently developed methods using crude antigens (8, 17 26) or antigens shared by gonococci and meningococci (18,19)

Because a definite structure the pilus, had now been demonstrated on virulent gonococci it was tempting to try this as antigen in a serological test for gonorrhoea In 1973 *Buchanan et al* (6) described promising results using gonococcal pili as antigen in a radioimmunoassay for quantitative determination of gonococcal antibodies in human sera.

Acta path. microbiol. scand. Sect. C, 85 123-130, 1977

PURIFICATION FROM EUGLOBULIN OF THE FIRST COMPONENT ($C\bar{I}$) OF COMPLEMENT AND ITS SUBCOMPONENTS BY HEPARIN-SEPHAROSE CHROMATOGRAPHY

G von Zeipel, Hanna-Stina Hanson and L. V. von Stedingk

The Central Microbiological Laboratory of the Stockholm County Council, Stockholm, Sweden

Zeipel, G. von, Hanson, H.-S. & Stedingk, L. V. von. Purification from euglobulin of the first component (CI) of complement and its subcomponents by heparin-Sepharose chromatography Acta path. microbiol. scand. Sect. C, 85 123-130 1977

Most of the CI material of euglobulin was adsorbed to heparin-Sepharose at an ionic strength of 0.265. After desorbtion at an ionic strength of 0.415 the CI material was found to be purified six to seven-fold. Highly purified subcomponents C1q, C1r and C1s were recovered at DEAE-Sephadex chromatography from such purified CI material after EDTA-treatment. Tests on isolated C1q, C1r and C1s disclosed in addition to the wellknown interaction between heparin and C1q an equally strong or even stronger interaction between heparin and C1s. Even C1r was adsorbed to heparin although by somewhat weaker ionic bonds

Key words CI subcomponents purification heparin-Sepharose chromatography

G von Zeipel, Virus Department of the Central Microbiological Laboratory of the Stockholm County Council, P O Box 177 S-101 22 Stockholm, Sweden.

Received 9 x.76 Accepted 3.xi.76

In a previous paper (*von Zeipel et al.* 1975) we reported on the purification of CI material from human euglobulin (EU) by heparin precipitation. After two successive precipitations the method regularly gave CI preparations purified six-fold at about 50 per cent yield with respect to the CI activity of the initial EU materials.

As a continuation of this work CI material and subcomponents of CI were in the present investigation purified from EU by chromatography on columns of heparin-Sepharose 4B. The latter were prepared as described by *Iverius* (1971) by coupling heparin containing serine or peptide residues to cyanobromide activated agarose. The chromatographic system was considered to enable more detailed studies on the interaction between heparin and CI material as well as between heparin and the subcomponents C1q, C1r and C1s. However the heparin preparation used was by necessity less pure than that employed in the batch method.

MATERIALS AND METHODS

Euglobulin (EU) was prepared from fresh human sera according to *Tamura & Nelson* (1968) and

11 *Kellogg D S Peacock W L Deacon, W E Brown L & Pirkle C I* *Neisseria gonorrhoeae* I Virulence genetically linked to clonal variation J Bact *85* 1274–1279 1963

12. *Kellogg D S Cohen I R Norins L C Schroeter A L & Reising G* *Neisseria gonorrhoeae* II Colonial variation and pathogenicity during 35 months *in vitro* J Bact *96* 596–605 1968

13 *Kolata G B* Gonorrhea More of a problem but less of a mystery Science *192* 244–247 1976

14 *Lightfoot R W Jr & Gotschlich E C* Gonococcal disease. Amer J Med. *56* 347–356 1974

15 *Lind I* Identification of *Neisseria gonorrhoeae* by means of fluorescent antibody technique. Acta path microbiol. scand. *70* 613–629 1967

16. *Lowry O H Rosebrough N J, Farr A L & Randall R J* Protein measurement with the folin phenol reagent J biol. Chem. *193* 265–275 1951

17 *Luoma M K Cross W R & Rudback J A* Radioimmunoassay for quantifying antibody to *N gonorrhoeae* in human sera. Brit. J vener Dis. *51* 387–391 1975

18. *Maeland J A* Serological cross-reactions of aqueous ether extracted endotoxin from *Neisseria gonorrhoeae* strains. Acta path. microbiol scand *77* 505–517 1969

19 *Maeland J A & Larsen B* Mercaptoethanol resistant human serum antibodies reacting with endotoxin from *Neisseria gonorrhoeae* Brit. J vener Dis. *51* 93–96 1975

20 *Novotny P Short J A & Walker P D* An electron-microscope study of naturally occurring and cultured cells of *Neisseria gonorrhoeae* J med. Microbiol. *8* 413–427 1975

21 *Novotny P & Turner W H* Immunological heterogeneity of pili of *Neisseria gonorrhoeae* J gen. Microbiol *89* 87–92 1975

22 *Peacock W L Jr Martin J E Jr., Thayer J D & Schroeter A L* Immunofluorescent detection of serum antibodies to the gonococcus among meningococcal carriers. Antimicrob. Ag Chemother 1964 p 649–651

23 *Ratnatunga C S* Evaluation of the gonococcal complement-fixation test. Brit J vener Dis. *47* 279–288 1971

24 *Reyn A* Laboratory diagnosis of gonococcal infections. Bull. Wld Hlth Org. *32* 449–469 1965

25 *Rodes C U & Ronald A R.* Comparison of three serological tests in gonococcal infection. Appl. Microbiol. *27* 695–698, 1974.

26 *Sandström E & Danielsson D* A survey of gonococcal serology In *Danielsson D Juhlin L & Mårdh P A* (Eds) Proceedings of the Symposium on Genital Infections and Their Complications. Almquist and Wiksell International Stockholm 1975 p. 233–239

27 *Swanson J Kraus S J & Gotschlich E C* Studies on gonococcus infection, I Pili and zones of adhesion their relation to gonococcal growth patterns. J exp Med. *134* 886–906, 1971

28. *Weinbach von R* Die Verwendbarkeit formolbehandelter Erythrocyten als Antigenträger in der indirekten Haemagglutination. Schweiz. Z. allg Path. *21* 1043–1052, 1958.

TABLE 1 *Composition of the E glob lin (EU) Preparation Used as Starting Material*

Material	Total amount of protein in mg	Total amounts in mg of the following components										Ratio	
		C1q	C1	C1s 1†	C1s 2‡	C1 IA	C3	C4	IgG	IgM	Albumin	C1q/C1 1†	C1q/C1 2‡
J derived from 5 ml serum	195	28.4 14.6%	n.d.	9 0 4.6%	13 4 6.9%	4 1 2.1%	3.7 1.9%	6 3 3.2%	63.8 32.7%	29.5 15.1%	1.3 0.7%	3 1	2.1

Per cent of the total amount of protein of the EU material.
C1s according to esterolytic assay
C1 according to immunodiffusion.
d = not done.

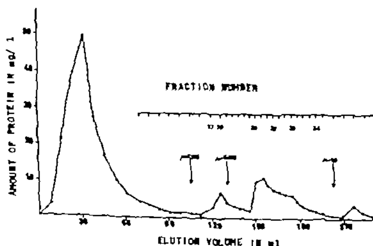


Fig 1 First cycle purification of euglobulin on heparin-Sepharose 4B Arrows mark the insertion of elution buffers of indicated ionic strengths For explanations see text

0 315 elution was inserted at an ionic strength of 0.415 and a speed of 20 ml per hour The column was then regenerated with a buffer μ = 1.0

The *main results are recorded in* Table 2. Out of the initial 195 mg of protein 13.5 per cent was eluted at an ionic strength of 0 415 (fractions 24–34) Apparently this peak contained most of the CI material of EU Thus C1q and CIs together made up for 63 per cent of the protein. The content of C1r was not determined. The *amount of* CIs was 69 per cent of the initial amount present in EU and 80 *per cent of the* total CIs recovery from the column. The amount of C1q, *on the other hand*, was only 36.7 per cent of the initial amount.

Obviously over 50 per cent of the initial amount of C1q but only 12 per cent of CIs passed the column before the ionic strength of 0.415 was reached. This *might* explain the shift during elution of the ratios (on weight basis) between the protein amounts of C1q and CIs whether the latter was determined by esterolysis or immunodiffusion. These ratios decreased from 3 and 2, respectively in EU (Table 1) to about 1 for the pool of fractions 31–34 (Table 2) Even within the peak at μ = 0 415 fixed ratios C1q/CIs were not obtained.

Laurell et al. (1970) as described earlier (von Zeipel et al. 1975). It was precipitated at pH 7.5 and dissolved in a 0.005 M phosphate buffer containing 0.3 M NaCl and 0.15 mM $CaCl_2$.

Synthetic amino acid esters. N-acetyl-L-tyrosine ethyl ester was from BDH Chemicals, Poole, England. N-acetyl-L-arginine methyl ester was from Cyclo Chemical Corp., Los Angeles, USA.

Esterolytic tests. Esterolysis was as previously (*von Zeipel et al.* 1974) measured with Titrator TTT and Autoburette ABU 12 from Radiometer (Copenhagen, Denmark). The reaction volume was 3 ml and included ester (0.02 M), esterase preparation and volume-compensating buffer (0.005 M phosphate buffer with 0.2 M NaCl). Calculations of units were based on initial 5 min periods of esterolysis.

Complement esterase (C1s). One unit of C1s was the amount that liberated 0.5 micromoles of H⁺ from 0.02 M N-acetyl-L-tyrosine ethyl ester (ATEe) in 15 min at pH 7.5 and 37° C. C1s was also measured by immunodiffusion (see below) and in some preparations according to the esterolytic methods of *Haines & Lepow* (1964), *Nagaki & Stroud* (1969) and *Bing* (1971).

Complement component C1r. C1r was assessed by measuring the hydrolysis of N-acetyl-L-arginine methyl ester (*Naff & Ratnoff* 1968) by column fractions devoid of C1s activity, i.e. lacking ATEe activity. One unit of C1r was, in analogy with C1s, the amount that liberated 0.5 micromoles of H⁺ from 0.02 M N-acetyl-L-arginine methyl ester in 15 min at pH 7.5 and 37° C.

C1q, C1s, C1 IA (C1 inactivator), C3, C9, IgG and IgM were assessed on EDTA treated samples by single radial immunodiffusion (*Mancini et al.* 1965) in plates containing 1 per cent agarose, 10 mM EDTA, 0.05 M Tris-glycine buffer (pH 8) and 0.15 M NaCl. Antisera from Behringwerke, Germany, were used at a concentration of 1 to 2 per cent. C3 and C4 were determined in Partigen plates from this company.

The reference preparation of C1q, prepared according to *Yonemasu & Stroud* (1971) was from Dr. S. E. Svehag, Odense University, Denmark. The reference preparation of C1 IA was from Behringwerke, Germany.

Heparin. Crude heparin (113 U.S.Ph units/mg) prepared from swine intestinal mucosa was obtained from Vitrum, Stockholm, Sweden. After purification as described by *Iverius* (1971) the activity of heparin had increased to 150 U.S.Ph units/mg.

Heparin-Sepharose 4B. 300 mg of purified heparin, dissolved in 10 ml of a buffer of pH 8 containing 0.1 M NaCl and 0.1 M $NaHCO_3$ was allowed to react with 15 g (dry weight) of CNBr activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) according to the manufacturers recommendations.

Columns were tested for leakage of heparin according to the method of *Yin et al.* (1973) employing reagents of the Sigma kit (Sigma, USA, Technical bulletin No. 870). The outcome was always negative.

Carbamylation of heparin-Sepharose material was carried out according to *Bjerrum et al.* (1973).

DEAE-Sephadex A25 was from Pharmacia Fine Chemicals, Uppsala, Sweden. DEAE-cellulose DE 52 was from Whatman & Balston, Maidstone, Kent, England.

Protein measurements were made with the Folin phenol reagent (*Lowry et al.* 1951) using bovine serum albumin as a standard. Protein in fractions containing EDTA, which is known to interact seriously with the Folin reagent (*Ji* 1972) was measured after precipitation by trichloroacetic acid (*Chase & Williams* 1968).

Preparative procedures were carried out at +4° C. Buffer solutions contained 0.01 per cent sodium azide.

RESULTS

Euglobulin

The various components specified in Table 1 constituted together 75 per cent of the protein content of EU as judged from our reference preparations. Yet C1r was not determined. Tests for C5 and C9 were negative. Using our final C1s preparation (1520 units/mg protein) as reference the content of C1s was 4.6 per cent according to the esterolytic assay and 6.9 per cent by immunodiffusion. This discrepancy was reproducible and large enough to suggest that part of the antigenically determined C1s of the starting material was esterolytically inactive.

First Cycle Chromatography

The EU material to be purified comprised 25 ml containing 7.8 mg protein per ml. It was dissolved in a buffer of 0.005 M phosphate, 0.25 M NaCl and 0.15 mM $CaCl_2$.

The material was pumped at a speed of 20 ml per hour into a pre-equilibrated column, 13 × 110 mm of heparin-Sepharose. This was washed with 40 ml starting buffer per hour (μ = 0.265, pH 7.5) until most of the proteins not absorbed had passed (Fig. 1). After a short additional washing at μ =

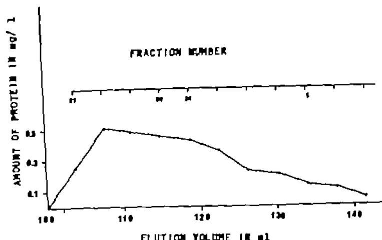


Fig. 2 Second cycle purification of CI material on heparin-Sepharose 4B. Elution peak at the ionic strength of 0.415. For explanations see text.

rial for 46 per cent of the initial CI amount.

C3 and C4 were not detectable in the eluate at $\mu = 0.415$

The total protein recovery from the column was 98.5 per cent of which 75 per cent passed with the starting buffer of $\mu = 0.265$

A similar purification experiment at pH 8.5 in 5 mM veronal buffer gave results almost identical with those described above.

Second Cycle Chromatography

A further purification of CI material was performed on the column used above but equilibrated with a 5 mM veronal buffer of pH 8.5 containing 0.15 mM $CaCl_2$ and 0.25 M NaCl. First cycle material of CI purified at pH 7.5 (fractions 26–34 Table 2) was equilibrated to the same buffer by dilution. The column was loaded and eluted as described above.

The main results are recorded in Table 3. Out of the initial amount of protein 66 per cent was eluted by $\mu = 0.415$ (Fig. 2). C1q and C1s constituted 67 per cent of this protein. C1r was not determined. It was again observed that the ratios C1q/C1s (initially 1.2–1.3) were high at the beginning of the elution (2.8–3.4) and decreased to 0.8–0.9.

The specific activity of C1s reached an average of 420 units in fractions 31–35 indicating a purification factor of 7 for CI material as compared to EU.

The total C1s recovery was 86 per cent.

CI inactivator was below detectable level after second cycle purification. The contents of IgG and IgM were, however, not lowered.

First cycle material run at a pH of 8.5 was also purified as above with almost identical results.

Isolation of Subcomponents C1q, C1r and C1s

In preliminary tests CI material chromatographed twice was treated with EDTA as described below and then loaded into columns of heparin-Sepharose at $\mu = 0.215$. With linear gradient buffers only a partial separation was achieved between C1q and C1s as they emerged in this order at an ionic strength of about 0.4. Subcomponents of CI were therefore isolated according to *Lepow et al.* (1963).

Twice chromatographed material of CI (corresponding to tubes 31–35 of Table 3) containing 3.26 mg C1q and 2.54 mg C1s out of 9.5 mg protein was split into the subcomponents C1q, C1r (initial content unknown) and C1s by EDTA treatment over night at +4 C at a pH of 7.5. The final ionic strength of the preparation was 0.13: 1 mM EDTA, 5 mM phosphate and 0.11 M NaCl.

TABLE 2 *Content of C1q, C1s, C1 IA, IgG and IgM in Fractions from First Cycle Purification of EU (see Fig. 1) on Heparin-Sepharose 4B*

Material Fraction No. according to Fig. 1	Total amount of protein in mg	Total amounts in mg of the following components							Ratio C1q/C1s	
		C1q	C1r	C1s 1†	C1s 2‡	C1 IA	IgG	IgM	1†	2‡
17–18	7.38	2.43 32.9%*	n.d.	0.17 2.3%	n.d.	0.42 5.7%	0.34 4.6%	0.35 4.7%	14.3	
24	3.91	1.89 48.5%	n.d.	0.31 7.9%	0.65 16.6%	0.12 3.1%	0.07 1.8%	0.14 3.6%	6.1	2.9
25	4.18	2.03 48.6%	n.d.	0.93 22.2%	n.d.	0.17 4.1%	0.08 1.9%	0.13 3.1%	2.2	
26	3.34	1.31 39.2%	n.d.	0.81 24.3%	n.d.	0.06 1.8%	0.06 1.8%	0.09 2.7%	1.6	
27–30	10.30	3.91 37.9%	n.d.	2.86 27.8%	3.95 38.3%	0.12 1.2%	0.10 1.0%	0.19 1.8%	1.4	1.0
31–34	4.67	1.26 27.0%	n.d.	1.28 27.4%	1.61 34.5%	0.01 0.2%	0.02 0.4%	0.07 1.5%	1.0	0.8

* Per cent of the amount protein of the respective fraction.
† ‡ n.d. see Table 1

TABLE 3 *Content of C1q, C1s, C1 IA, IgG and IgM in Fractions from Second Cycle Purification of EU (see Fig. 2) on Heparin-Sepharose 4B*

Material	Total amount of protein in mg	Total amounts in mg of the following components							Ratio C1q/C1s	
		C1q	C1r	C1s 1†	C1s 2‡	C1 IA	IgG	IgM	1†	2‡
Fractions 26–34 of first cycle Table 2	18.32	6.48 35.4%*	n.d.	4.95 27.0%	5.31 29.0%	0.19 1.0%	0.18 1.0%	0.35 1.9%	1.3	1.2
Fraction No. according to Fig. 2										
27	0.97	0.55 56.7%	n.d.	0.16 16.5%	0.2 20.6%	–	0.01 1.0%	0.03 3.1%	3.4	2.8
28	1.97	0.80 40.6%	n.d.	0.48 24.4%	0.63 32.0%	–	0.01 0.5%	0.03 1.5%	1.7	1.3
29	1.89	0.78 41.3%	n.d.	0.53 28.0%	0.61 32.3%	–	0.01 0.5%	0.04 2.1%	1.5	1.3
30	1.79	0.69 38.6%	n.d.	0.53 29.6%	0.75 41.9%	–	0.01 0.6%	0.04 2.2%	1.3	0.9
31–35	5.59	1.73 31.0%	n.d.	1.89 33.8%	2.16 38.6%	–	0.03 0.5%	0.14 2.5%	0.92	0.8

* † ‡ n.d. see Table 1
– = negative.

The specific activity of C1s was 104 units per mg at the beginning of the elution at $\mu = 0.415$ (fraction 24) and reached a mean value of 366 units in fractions 26–34. In four of these fractions activities of 380 units per mg were recorded. As the initial C1s activity was 61 units per mg EU protein this would indicate a six fold purification of C1 mate-

complexes. This is indicated by the absence of constant ratios in the eluted fractions between the amounts of C1q and C1s irrespective of whether the C1s amount was calculated on the basis of esterolytic determinations on C1 IA free fractions (after second cycle chromatography) or from immunodiffusion.

Heparin-Sepharose chromatography followed by DEAE chromatography permitted the simultaneous isolation of highly purified C1q, C1r and C1s. The specific esterolytic activity of the latter component was notably higher than found by previous authors.

In addition to the wellknown interaction between C1q and heparin an equally strong or even stronger interaction was found between C1s and heparin. Even C1r was adsorbed to heparin although at a lower ionic strength than C1q and C1s. The interactions between heparin and C1s and C1r were probably not due to residual positive charges of the heparin used as they also took place after carbamylation of the aminogroups of the heparin bound to Sepharose.

After this paper was submitted for publication *Loos et al.* (Immunochemistry 13 789–791 1976) have published an interaction between heparin and C1s occurring at presumably positive site for C4 or C2 on C1s. Such site may be responsible for the interaction in our case

The skilful technical assistance of M. *N Broberg* is gratefully acknowledged

REFERENCES

1. *Assimeh S N Bing, D H & Painter R. H* A simple method for the isolation of the subcomponents of the first component of complement by affinity chromatography J Immunol. *113* 225–234 1974
2. *Bing D H* Purification of the first component of human complement by affinity chromatography on human γ-globulin linked to Sepharose J Immunol. *107* 1243–1249 1971
3. *Bjerrum O J I gild A Lowenstein H & Weeke B.* Carbamylated antibodies used for quantitation of human IgG. A routine method. I *Axelsen N H Kroll, J & Weeke B* (Eds) A manual of quantitative immunoelectrophoresis Methods and Applications. Scand, J Immunol. Suppl. No. 1 1973 p. 148
4. *Chase M W & Williams C. A.:* Protein analysis. In *Williams, C A. & Chase M W* (Eds.): Methods in immunology and immunochemistry Vol. 2 Academic Press, New York and London, 1968 p. 275
5. *Colten H R., Borsos T & Rapp H J.:* Reversible loss of activity of the first component of complement (C'1) as function of ionic strength. J Immunol. *100* 799–807 1968 a.
6. *Colten H R., Borsos T & Rapp H J.:* Ultracentrifugation of the first component of complement effects of ionic strength. J Immunol. *100* 808–813 1968 b.
7. *Haines A L. & Lepow I H.:* Studies on human C1-esterase. I Purification and enzymatic properties. J Immunol 92 456–467 1964
8. *I axius P.-H* Coupling of glycosaminoglycans to agarose beads (Sepharose 4 B) Biochem. J *124* 677–683 1971
9. *Ji, T H.:* Interference by detergents, chelating agents, and buffers with the Lowry protein determination. Anal. Biochem. 52 517–521 1973
10. *Lau B, A-B Mårtensson U & Sjöholm, A G* C1 subcomponent complexes in normal and patholog cal sera studied by crossed immunoelectrophoresis. Acta path. microbiol. scand. Sect. C, *84* 455–464 1976.
11. *Lepow I H Naff G B., Todd E. W., Pensky J & Hinz C F* Chromatographic resolution of the first component of human complement into three activities. J Exp. Med. *117* 983–1008, 1963
12. *Lowry O H Rosebrough N J., Farr A L. & Randall, R. J* Protein measurement with the Folin phenol reagent. J Biol. Chem. *193* 265–275 1951
13. *Mancini, G Carbonara A O & Heremans J F.:* Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 2 235–254 1965
14. *Naff G B & Ratnoff O D.:* The enzymatic nature of C1 Conversion of C1s to C1 esterase and digestion of amino acid esters by C1r J Exp. Med. *128* 571–593 1968.
15. *Nagaki K. & Stroud R. M.:* The relationship of the hemolytic activity of active C1s to its TAMe esterase action A new method of purification and assay J Immunol. *102* 421 430 1969
16. *Nagaki, K & Stroud R M.:* Immunochemical quantitation of human C1 esterase (C1s) concentration in normal serum and purification yields J Immunol. *105* 170–174 1970.
17. *Tamura, N & Nelson, R. A.:* The purification and reactivity of the first component of complement from guinea pig, human and canine sera J Immunol. *101* 1333–1345, 1968.

The material was pumped at a speed of 8 ml per hour into a column of DEAE-Sephadex A 25 13 × 120 mm. Elution was accomplished in 6 hours at a speed of 30 ml per hour by a linear gradient rising from μ = 0 13 to a final ionic strength of 0.5 (1 mM EDTA 5 mM phosphate and 0 48 M NaCl)

CIq passed through the column with the starting buffer

A peak comprising 74 per cent of the eluted CIr activity occurred at ionic strengths 0 18 to 0.21 The highest specific activity obtained was 1000 units per mg protein. Calculated on the basis of this purity level a total amount of 1 7 mg CIr was recovered

76 per cent of the eluted CIs activity was located in a peak between μ = 0.3 and μ = 0.33 The highest specific activity was 1320 units per mg protein representing a 22 fold purification with respect to the esterase activity of EU Approximating this preparation as representative of pure CIs a total amount of 1 6 mg CIs was recovered.

Separation on DEAE cellulose gave closely similar results.

The solutions of CIq CIr and CIs, respectively could be concentrated 5 times with about 90 per cent yield by adsorption on carbamylated heparin-Sepharose columns at the ionic strengths 0 1 (CIr) 0 2 (CIq and CIs) followed by elution at μ = 0 6 (1 mM EDTA, 5 mM phosphate and 0.58 M NaCl)

The final preparation of CIq (1 5 mg) contained 5 to 10 per cent more CIq per mg protein compared to the standard preparation used in immunodiffusion tests. It lacked CIr and CIs activities but still contained small amounts of IgG (0 1 per cent) and IgM (1.5 per cent)

The final preparation of CIs was taken as a standard in our esterolytic and immunodiffusion assays of CIs. It contained no detectable amounts of CIq IgG or IgM In parallel determinations our value for the specific activity (1320 U/mg protein) corresponded to 1460 U/mg according to the method of *Haines & Lepow* (1964) to 3010 U/mg according to the method of *Bing* (1971) and to 4200 U/mg according to the method of *Nagaki & Stroud* (1969) The highest values reported by *Haines & Lepow* was 608 U/mg *Assimeh et al* (1974) 570 U/mg and *Nagaki & Stroud* (1970) 2674 U/mg.

The final preparation of CIr was contaminated by trace amounts of CIq but was free from detectable amounts of CIs, IgG and IgM To our knowledge no value for the specific esterolytic activity of highly purified CIr is previously reported in the literature.

DISCUSSION

The term CI material is used in the present investigation to denote complexes of CIq, CIr and CIs not necessarily identical to the first component of complement (CI) as found in human serum The reason for this is our use of such elevated ionic strengths as *Colten et al.* (1968 a, 1968 b) have found to be able to dissociate the CI complex into subunits not corresponding to CIq CIr and CIs. Furthermore our EU may have contained complexes such as CIr-CIs and/or CIr-CIs-CI IA which were shown by *Laurell et al.* (1976) to be present also in normal human sera.

The ionic strength at which most of the CI material of EU was adsorbed to heparin-Sepharose was as high as 0.265 The CI material was furtheron desorbed at an ionic strength of 0 415 apparently about six-fold purified as judged from CIs determinations. The ionic strengths regulating the interaction between CI material and heparin-Sepharose were thus closely similar to those used at the purification of CI material by heparin precipitation (*von Zeipel et al* 1975) which also gave six fold purified CI material. Even a second cycle of chromatography did not free the CI material from small amounts of IgG and IgM

At chromatography of EU an excess of probably uncomplexed CIq was eluted from the heparin-Sepharose column prior to the desorption of the CI material. The CI material was probably heterogenous and may have contained various not further studied

complexes. This is indicated by the absence of constant ratios in the eluted fractions between the amounts of C1q and C1s irrespective of whether the C1s amount was calculated on the basis of esterolytic determinations on C1 IA free fractions (after second cycle chromatography) or from immunodiffusion.

Heparin-Sepharose chromatography followed by DEAE chromatography permitted the simultaneous isolation of highly purified C1q, C1r and C1s. The specific esterolytic activity of the latter component was notably higher than found by previous authors.

In addition to the wellknown interaction between C1q and heparin an equally strong or even stronger interaction was found between C1s and heparin. Even C1r was adsorbed to heparin although at a lower ionic strength than C1q and C1s. The interactions between heparin and C1s and C1r were probably not due to residual positive charges of the heparin used as they also took place after carbamylation of the aminogroups of the heparin bound to Sepharose.

After this paper was submitted for publication *Loo et al.* (Immunochemistry 13 789–791 1976) has published an interaction between heparin and C1 occurring at presumably positive site for C4 or C2 on C1s. Such sites may be responsible for the interaction in our case.

The skilful technical assistance of Mr *N Brobäng* is gratefully acknowledged.

REFERENCES

1. *Assimeh, S N Bing, D H & Painter R. H* A simple method for the isolation of the subcomponents of the first component of complement by affinity chromatography J Immunol. 113 225–234 1974
2. *Bing, D H* Purification of the first component of human complement by affinity chromatography on human γ-globulin linked to Sepharose. J Immunol 107 1243–1249 1971
3. *Bjerrum O J I gild A Laurentsin, H & Weeke B.* Carbamylated antibodies used for quantitation of human IgG A routine method. I *Axelsen N. H., Kroll, J & Weeke B.* (Eds.) A manual of quantitative immunoelectrophoresis Methods and Applications. Scand. J Immunol. Suppl. No 1 1973 p. 148.
4. *Chase M W & Williams C A.* Protein analysis. In *Williams, C. A. & Chase M W* (Eds.) Methods in immunology and immunochemistry Vol. 2, Academic Press, New York and London, 1968 p. 273
5. *Colten H R., Bondes T & R pp H J.:* Reversible loss of activity of the first component of complement (C'1) as function of ionic strength J Immunol. *100* 799–807 1968 a.
6. *Colten, H R., Borsos T & Rapp H J.* Ultracentrifugation of the first component of complement: effects of ionic strength. J Immunol. 100 808–813 1968 b.
7. *Haines A. L. & Lepow I H.* Studies on human C1-esterase. I. Purification and enzymatic properties. J Immunol. *92* 456–467 1964
8. *I ems P H.* Coupling of glycosaminoglycans to agarose beads (Sepharose 4 B) Biochem. J *124* 677–685, 1971
9. *Ji, T H* Interference by detergents, chelating agents, and buffers with the Lowry protein determination. Anal. Biochem 52 517–521 1973
10. *Laurell A.-B Mårtensson U & Sjöholm A. G* C1 subcomponent complexes in normal and patholog cal sera studied by crossed immunoelectrophoresis. Acta path. microbiol. scand. Sect. C, *84:* 455–464 1976.
11. *Lepow I H Naff G B., Todd E. W., Pensky J & Hinz C. F* Chromatographic resolution of the first component of human complement into three activities. J Exp. Med. *117* 983–1008, 1963
12. *Lowry O H., Rosebrough, N J Farr A L. & Randall, R J.* Protein measurement with the Folin phenol reagent. J Biol. Chem. *193:* 265–275 1951
13. *Mancini G., Carbonara A O & Heremans, J F.* Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 2 235–254 1965
14. *Naff G B. & Ratnoff O D.:* The enzymatic nature of C1r Conversion of C1s to C1 esterase and digestion of amino acid esters by C1 J Exp. Med. *128* 571 593 1968.
15. *Nagaki K. & Stroud R. M* The relationship of the hemolytic activity of active C1 to its TAMe esterase action A new method of purification and assay J Immunol. *102* 421–430, 1969
16. *Nagaki K. & Stroud R M.:* Immunochemical quantitation of human C1 esterase (C1s) concentration in normal serum and purification yields J Immunol. *105* 170–174 1970.
17. *Tamura N & Nelson R. A.:* The purification and reactivity of the first component of complement from guinea pig, human and canine sera. J Immunol. *101* 1333–1345, 1968.

The material was pumped at a speed of 8 ml per hour into a column of DEAE-Sephadex A 25 13 × 120 mm Elution was accomplished in 6 hours at a speed of 30 ml per hour by a linear gradient rising from μ = 0 13 to a final ionic strength of 0.5 (1 mM EDTA 5 mM phosphate and 0 48 M NaCl)

Clq passed through the column with the starting buffer

A peak comprising 74 per cent of the eluted CIr activity occurred at ionic strengths 0 18 to 0 21 The highest specific activity obtained was 1000 units per mg protein Calculated on the basis of this purity level a total amount of 1 7 mg CIr was recovered

76 per cent of the eluted CIs activity was located in a peak between μ = 0.3 and μ = 0.33 The highest specific activity was 1320 units per mg protein representing a 22 fold purification with respect to the esterase activity of EU Approximating this preparation as representative of pure CIs a total amount of 1 6 mg CIs was recovered.

Separation on DEAE cellulose gave closely similar results.

The solutions of Clq CIr and CIs, respectively could be concentrated 5 times with about 90 per cent yield by adsorption on carbamylated heparin-Sepharose columns at the ionic strengths 0 1 (CIr) 0 2 (Clq and CIs) followed by elution at μ = 0 6 (1 mM EDTA 5 mM phosphate and 0 58 M NaCl)

The final preparation of Clq (1.5 mg) contained 5 to 10 per cent more Clq per mg protein compared to the standard preparation used in immunodiffusion tests. It lacked CIr and CIs activities but still contained small amounts of IgG (0 1 per cent) and IgM (1.5 per cent)

The final preparation of CIs was taken as a standard in our esterolytic and immunodiffusion assays of CIs. It contained no detectable amounts of Clq IgG or IgM In parallel determinations our value for the specific activity (1320 U/mg protein) corresponded to 1460 U/mg according to the method of *Haines & Lepow* (1964) to 3010 U/mg according to the method of *Bing* (1971) and to 4200 U/mg according to the method of *Nagaki & Stroud* (1969) The highest values reported by *Haines & Lepow* was 608 U/mg, *Assimeh et al.* (1974) 570 U/mg and *Nagaki & Stroud* (1970) 2674 U/mg

The final preparation of CIr was contaminated by trace amounts of Clq but was free from detectable amounts of CIs, IgG and IgM To our knowledge no value for the specific esterolytic activity of highly purified CIr is previously reported in the literature.

DISCUSSION

The term CI material is used in the present investigation to denote complexes of Clq, CIr and CIs not necessarily identical to the first component of complement (CI) as found in human serum The reason for this is our use of such elevated ionic strengths as *Colten et al* (1968 a 1968 b) have found to be able to dissociate the CI complex into subunits not corresponding to Clq CIr and CIs. Furthermore our EU may have contained complexes such as CIr-CIs and/or CIr-CIs-CI IA which were shown by *Laurell et al* (1976) to be present also in normal human sera

The ionic strength at which most of the CI material of EU was adsorbed to heparin-Sepharose was as high as 0 265 The CI material was furtheron desorbed at an ionic strength of 0 415 apparently about six-fold purified as judged from CIs determinations. The ionic strengths regulating the interaction between CI material and heparin-Sepharose were thus closely similar to those used at the purification of CI material by heparin precipitation (*von Zeipel et al.* 1975) which also gave six fold purified CI material. Even a second cycle of chromatography did not free the CI material from small amounts of IgG and IgM

At chromatography of EU an excess of probably uncomplexed Clq was eluted from the heparin-Sepharose column prior to the desorption of the CI material. The CI material was probably heterogenous and may have contained various not further studied

Acta path. microbiol. scand. Sect. C, 85: 131–141, 1977

EFFECT OF EXPERIMENTAL TRICHINOSIS ON UNRELATED HUMORAL AND CELL MEDIATED IMMUNITY

INGER LJUNGSTRÖM and GUNNEL HULDT

National Bacteriological Laboratory, 105 21 Stockholm, Sweden

Ljungström, I. & Huldt, G. Effect of experimental trichinosis on unrelated humoral and cell mediated immunity. Acta path. microbiol. scand. Sect. C, 85: 131–141, 1977.

Immune responses to unrelated antigens were studied during the course of *Trichinella spiralis* infection in mice. A transitory depression of the IgM response to the thymus dependent antigen, sheep erythrocytes, was seen three weeks after infection and this effect was more pronounced after challenge. A depression of the IgG response also was observed, however not until 6 weeks after infection. The humoral response to the thymus independent antigen, polyvinyl pyrrolidone showed a slight increase which was most evident in challenged mice. The nonspecific cellular immune response, as measured by the split heart allograft technique, showed a profound and longlasting depression. This effect was most pronounced one week after inoculation, when the parasite resides mainly in the intestine. The time at which the nonspecific cellular immunity was most depressed coincides with the period of strong specific cell mediated immunity. It is also shown that experimental trichinosis in CBA mice is accompanied by profound but transient changes in the thymus reflected mainly as a depletion of cortical thymocytes. These changes were not due to corticosteroids or malnutrition. There is reason to believe that thymic depletion is of significance for the depressed antibody response, but not for the prolonged survival of allografts. These findings indicate that *T. spiralis* affects the nonspecific immunity at different levels.

Key words: Experimental trichinosis; effect of; humoral immunity; cell mediated immunity.

I. Ljungström, National Bacteriological Laboratory, 105 21 Stockholm, Sweden.

Received 26.v.76. Accepted 16.xi.76.

Over the last few years evidence has accumulated to show that parasitic infections can affect the immune responses of the host to unrelated antigens. Most work has been carried out on protozoan infections (15, 18, 20, 29, 30, 33). Studies of immunodepression caused by helminths have been mainly concerned with *Trichinella spiralis* in mice, in which the humoral immune responses to unrelated antigens have been measured (3, 7, 13, 28). Recently two studies (12, 27) suggested a transitory depression of the antibody response to sheep erythrocytes, a thymus dependent antigen. The effect of *T. spiralis* infection on cellular immunity as measured by graft survival, has been carried out on a single occasion during the course of infection (14, 35). The results show a prolonged survival time of skin grafts in infected mice compared with uninfected. The purpose of this study was to investigate the effect of *T. spiralis* on both the humoral and cellular immune res-

9*

18. *Vroon D H Schultz D R & Zarco R M* The separation of nine components and two inactivators of components of complement in human serum. Immunochemistry 7 43–61 1970

19 *Yin E. T Wessler S & Butler J.* Plasma heparin a unique practical, submicrogram sensitive assay J Lab. Clin. Med 81 298–310 1973

20 *Yonemasu K & Stroud R M* Clq rapid purification method for preparation of monospecific antisera and for biochemical studies J Immunol. 106 304–313 1971

21 *von Zeipel G Hanson H-S & von Stedingk L. V* Purification by ionic precipitation of the first component of human complement and complement esterase. Acta path. microbiol. scand. Sect. B 82 590–592, 1974

22. *von Zeipel G Hanson H-S & von Stedingk, L. V.* Purification from euglobulin of the first component (C1) of complement by heparin, suramin and Liquoid precipitation. Acta path. microbiol. scand. Sect. C, 83 241–243 1975.

recommended by *Denham & Martinez* (10). Uninfected, transplanted and methyridine treated mice were used as controls.

Weight determination and host pathology. Total body and thymus weight of infected and uninfected mice were determined 3, 7, 11, 21, 28 and 38 days after infection. Thymus tissues were fixed in 10 per cent buffered formalin and examined by conventional histopathological techniques.

Adrenalectomy. Thirty infected mice and thirty age-matched normal mice were adrenalectomized after laparotomy under ether anaesthesia 19 days after infection. Tw. days later the infected mice were challenged with 500 larvae. Groups of 24 normal mice and 30 intact and challenged mice served as control.

RESULTS

The response to SRBC after primary infection. This was measured after administration of antigen 1, 3 and 6 weeks after infection.

No significant change in antibody response or in the number of IgM and IgG plaque forming cells was demonstrated when SRBC was given 1 week after infection.

However, when SRBC was given 3 weeks after infection the IgM response was lower in infected mice than in controls. Four days after immunization the number of IgM plaque forming cells was significantly lower ($p<0.05$) in infected mice. The agglutinating antibody and haemolytic levels also appeared to be lower, but the difference was not statistically significant. Thirteen days after immunization neither antibody titres nor the number of plaque forming cells differed significantly in infected mice and controls. Thus no difference in the IgG response could be demonstrated.

Immunization with SRBC 42 days after infection was accompanied by a lower number of IgM plaque forming cells 4 days after immunization, while 13 days after immunization a significantly decreased number of IgG plaques ($p<0.02$) was measured.

The eff ct of challenge on the response to SRBC. Antibody responses to red cells given 3 days after a challenge infection are shown in Table 1. Four days after immunization, the agglutinin antibody level ($p<0.01$) as well as the number of IgM plaque forming cells ($p<0.05$) were significantly lower in infected mice than in controls. The level of haemolytic antibodies was also lower, but the difference was not statistically significant.

Thirteen days after immunization the level of haemolytic antibodies was significantly lower in infected mice ($p<0.02$), but no difference between infected and controls was demonstrated with regard to agglutinins or number of plaque forming cells (Table 1).

The antibody response to PVP. CBA mice were found to be low responders to PVP immunization. Thus, the experiment was repeated in A/Sn mice known to be high responders to PVP (2). The antibody response to PVP was measured 1, 2 and 4 weeks after immunization which was carried out 1 and 2 weeks after primary infection and 3 days after challenge. The results obtained in all experiments with PVP showed that infected animals tended to have higher antibody level, but the difference was not statistically significant.

Survival time of heart grafts transplanted at different times after infection. The *T. spiralis* infected mice were transplanted on the day of infection and after 1 week (intestinal stage). Mice were also transplanted on weeks 2 (migration stage), 3 (early muscular stage) and 11 (late muscular stage) after infection.

T. spiralis did not change the survival time of grafts given on the same day as the infection. The rejection of the transplants given at the other times, however, was significantly delayed ($p<0.001$). As shown in Fig. 1, the most pronounced delay of graft rejection was obtained in mice grafted one week after infection.

Survival time of allografts transplanted in challenged mice. The mice were challenged three weeks after infection with 500 larvae. Some were challenged and transplanted on the same day and others transplanted one week after challenge. As seen in Fig. 2, a significantly delayed rejection of the grafts was observed in both groups ($p<0.001$). However, the prolongation was more pronounced in mice transplanted one week after

ponses of mice to unrelated antigens at different stages of the infection

To study the antibody response, groups of mice were infected with *T spiralis* and subsequently injected with sheep red blood cells (SRBC, thymus dependent) or polyvinyl pyrrolidone (PVP thymus independent) Levels of humoral antibody to SRBC were found by measuring serum agglutinins and haemolysins the number of cells producing antibody was determined by a modified Jerne haemolytic plaque assay Antibodies to PVP were determined by indirect haemolysis.

To investigate cell mediated immunity during the course of infection it was decided to use the split heart allograft technique. It is well known that graft rejection is mainly due to cell mediated immunity and that the survival time of grafts gives a reasonably good measurement of such immunity The method chosen is sensitive simple and objective.

Total body weight and the thymus weight were followed over the observation period. Adrenalectomized mice were used to determine whether corticosteroids were responsible for the thymus reduction. The technical details of the procedures are given below

MATERIALS AND METHODS

Animals Male mice of either CBA (H 2^k) or A/Sn strain were used in the experiments They were 8 weeks old when first infected. Donor hearts were obtained from 12 36 hours old C57BL (H 2^b) mice

Parasite and infection The strain of *T spiralis* used has been maintained at the National Bacteriological Laboratory since 1946 by passage through laboratory rats. Larvae were obtained from the muscles of infected Sprague Dawley rats by HCl pepsin digestion (25) The larvae were washed resuspended in saline and administered by means of a stomach tube. All the mice were infected on day 0 with 500 larvae and some groups were challenged with 500 larvae 3 weeks after initial infection.

Antigens and immunization Sheep red blood cells (SRBC) were obtained from the same animal for all experiments. They were stored in Alsever's solution at 4 C for one week before use. The cells were washed 4 times in isotonic balanced salt solution, pH 7 4 (BSS) resuspended to 25 per cent and 0.2 ml of the suspension was injected intraperitoneally Polyvinyl pyrrolidone (PVP) – K90 mol.wt. 360 000 was obtained from Fluka AG Switzerland. The mice were given a single intravenous injection of 5 µg of antigen in BSS

Immunological Tests

Agglutinating antibodies Antisera were serially diluted in BSS and each dilution was mixed with an equal volume (25 µl) of 2 per cent washed SRBC. Agglutination was measured after 45 minutes at 37 C. Sera from infected but not immunized mice were used as controls.

Haemolytic antibodies Serial dilutions of antisera made in BSS were incubated with equal volumes (25 µl) of 1.5 per cent SRBC and complement. Control sera from infected but unimmunized mice were similarly treated. Haemolysis was determined after 45 minutes at 37°C.

Antibody producing cells The number of cells producing antibody was determined by a modified Jerne haemolytic plaque assay (4 22) The direct plaque assay was carried out to find the number of cells producing mainly IgM. To determine the IgG producing cells the indirect plaque assay was carried out (11 32) Control cells were obtained from normal mice and mice infected but not immunized.

Indirect haemolysis Serum antibodies against PVP were determined by indirect haemolysis according to *Andersson* (1) Indicator erythrocytes were prepared by incubating tanned SRBC in BSS containing 0 1 mg PVP (K19 mol.wt. 10,000) per ml. The labelled red cells were washed in BSS and were used to test for haemolysis as described above

Transplantation. Heart grafts were performed as described by *Huff et al.* (19) and *Judd & Trentin* (23) with the modification of *Sieburg & Schilling* (34) Hearts from newborn C57BL mice were sectioned along the ventricular septum and each half inserted into a subcutaneous pouch on the dorsal part of the ear of the recipient mouse. The survival of the transplant was monitored by measuring the electric activity with a Tektronix 410 cardioscope. As controls, uninfected transplanted mice were used The activity of the host hearts was also registered. This activity pattern was quite different from the transplanted hearts and did not confuse measurements of the latter

Irradiation The larvae were sterilised with 3990 rads, generated in a Siemens X ray machine at 15 mA 220 V for 11 min at a rate of 362 R/min (9)

Methyridine treatment At four and a half days after infection the mice were injected subcutaneously with 0 1 ml/10 g mouse of a 5 per cent solution of methyridine This dose was given three times, at 0, 6 and 24 hours after the first injection as

recommended by *Dunham & Martinez* (10) Uninfected, transplanted and methyridine treated mice were used as controls.

Weight determination and histopathology Total body and thymus weight of infected and uninfected mice were determined 5 7 11 21 28 and 38 days after infection. Thymus tissues were fixed in 10 per cent buffered formalin and examined by conventional histopathological techniques.

Adrenalectomy Thirty infected mice and thirty age-matched normal mice were adrenalectomized after laparotomy under ether anaesthesia 19 days after infection. Two days later the infected mice were challenged with 500 larvae. Groups of 24 normal mice and 30 intact and challenged mice served as control.

RESULTS

The response to SRBC after primary infection This was measured after administration of antigen 1 3 and 6 weeks after infection.

No significant change in antibody response or in the number of IgM and IgG plaque forming cells was demonstrated when SRBC was given 1 week after infection.

However when SRBC was given 3 weeks after infection the IgM response was lower in infected mice than in controls. Four days after immunization the number of IgM plaque forming cells was significantly lower ($p<0.05$) in infected mice. The agglutinating antibody and haemolytic levels also appeared to be lower but the difference was not statistically significant. Thirteen days after immunization neither antibody titres nor the number of plaque forming cells differed significantly in infected mice and controls. Thus no difference in the IgG response could be demonstrated.

Immunization with SRBC 42 days after infection was accompanied by a lower number of IgM plaque forming cells 4 days after immunization, while 13 days after immunization a significantly decreased number of IgG plaques ($p<0.02$) was measured.

The effect of challenge on the response to SRBC Antibody responses to red cells given 3 days after a challenge infection are shown in Table 1 Four days after immunization, the agglutinin antibody level ($p<0.01$) as well as the number of IgM plaque forming cells ($p<0.05$) were significantly lower in infected mice than in controls. The level of haemolytic antibodies was also lower but the difference was not statistically significant.

Thirteen days after immunization the level of haemolytic antibodies was significantly lower in infected mice ($p<0.02$) but no difference between infected and controls was demonstrated with regard to agglutinins or number of plaque forming cells (Table 1)

The antibody response to PVP CBA mice were found to be low responders to PVP immunization. Thus, the experiment was repeated in A/Sn mice known to be high responders to PVP (2) The antibody response to PVP was measured 1 2 and 4 weeks after immunization which was carried out 1 and 2 weeks after primary infection and 3 days after challenge. The results obtained in all experiments with PVP showed that infected animals tended to have higher antibody level, but the difference was not statistically significant.

Survival time of heart grafts transplanted at different times after infection. The *T spiralis* infected mice were transplanted on the day of infection and after 1 week (intestinal stage) Mice were also transplanted on weeks 2 (migration stage) 3 (early muscular stage) and 11 (late muscular stage) after infection.

T spiralis did not change the survival time of grafts given on the same day as the infection. The rejection of the transplants given at the other times, however was significantly delayed ($p<0.001$) As shown in Fig. 1 the most pronounced delay of graft rejection was obtained in mice grafted one week after infection.

Survival time of allografts transplanted in challenged mice The mice were challenged three weeks after infection with 500 larvae. Some were challenged and transplanted on the same day and others transplanted one week after challenge. As seen in Fig 2, a significantly delayed rejection of the grafts was observed in both groups ($p<0.001$) However the prolongation was more pronounced in mice transplanted one week after

TABLE 1 *The Antibody Response to Sheep Erythrocytes (SRBC) in CBA Mice Infected with 500* T spiralis *Larvae at Day 0 and Challenged 3 Weeks Later with 500 Larvae and in Age matched Normal Mice*

Antibody response 4 days after immunization (A)

Challenge days after inf	Immunization days after inf	Status	No. of animals	Direct PFC per 10^6 cells ± S.E.	t p	Agglutinin titre ± S.E	t p	Haemolytic titre ± S.E	t p
21	24	Normal *T spiralis*	10 10	162 ± 21 93 ± 20	2.387 0 05*	111 ± 21 42 ± 9	3 047 0 01**	239 ± 42 169 ± 34	1.292 0.3

Antibody response 13 days after immunization (B)

Challenge days after inf	Immunization days after inf	Status	No of animals	Indirect PFC per 10^6 cells ± S E	t p	Agglutinin titre ± S E	t p	Haemolytic titre ± S E.	t p
21	24	Normal *T spiralis*	10 10	21 ± 8 29 ± 5	0 838 0.5	119 ± 16 104 ± 12	0 788 0.5	97 ± 23 32 ± 5	2 775 0 02*

The controls which consisted of (I) unimmunized, infected mice and (II) unimmunized and uninfected mice produced only negligible numbers of plaque forming cells (PFC) In the serological tests they were all negative. The numbers of indirect PFC (A) and indirect PFC (B) were both just above the controls.

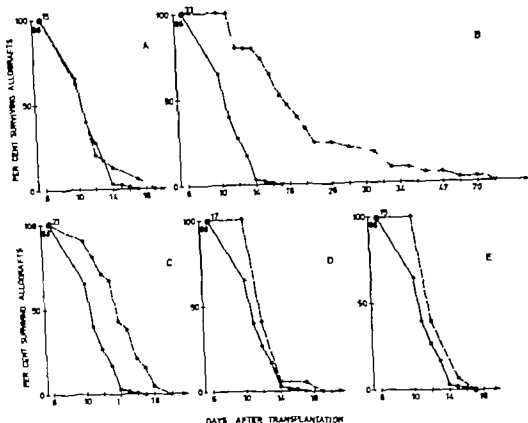


Fig 1 Per cent surviving allografts in CBA mice infected with 500 *T spiralis* larvae (O--O) and in age matched normal mice (●—●). Transplantation performed on the day of infection (A) and on weeks 1 (B) 2 (C) 3 (D) and 11 (E) after infection. The number of transplants is shown at the top of each figure

challenge than in mice challenged and transplanted on the same day

On the other hand, the allograft survival time was significantly longer in mice challenged and transplanted three weeks after primary infection compared to non-challenged mice transplanted at the same time after primary infection ($p<0.01$)

However it should be noticed that grafts given to challenged mice one week after re infection did not result in the same marked prolongation of the survival time as that observed in primary infected mice transplanted one week after infection.

Effect of curtailed infection on allograft survival time Methyridine an antihelmintic drug with selective effect on adult worms (5 8 31) was given to mice four and a half days after infection. This treatment destroys the adults and prevents the next stages of the infection, as the production of larvae starts about 5 days after infection (8) The treated mice were transplanted one week after infection.

Also under these conditions, the graft rejection was found to be delayed as compared with that in uninfected mice, whether untreated or treated with methyridine ($p<0.001$) (Fig. 3 A)

Furthermore, in mice with a curtailed infection, the grafts survived longer than in mice infected with irradiated larvae. On the

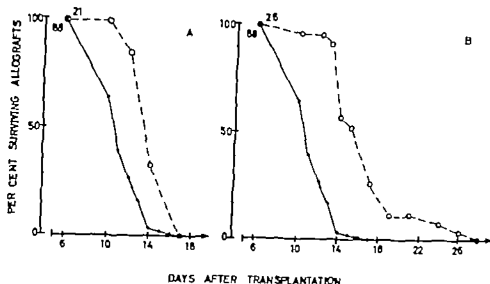


Fig 2 Per cent surviving allografts in CBA mice infected with 500 *T spiralis* larvae on day 0 and challenged 3 weeks later (O--O) and in age-matched controls (●—●) The number of transplants is shown at the top of each figure

A. Transplantation performed on the day of challenge

B. Transplantation performed 1 week after challenge.

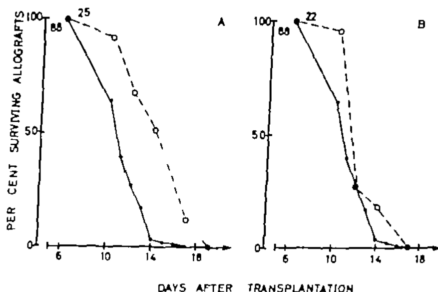


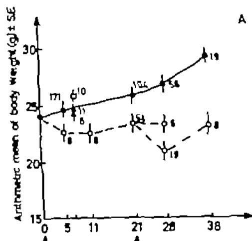
Fig 3 A. Per cent surviving allografts in CBA mice treated with methyridine 4.5 days after infection with 500 *T spiralis* larvae (O--O) methyridine treated normal mice (+   +) and in age-matched normal mice (●—●) Transplantation performed 1 week after infection. The number of transplants is shown at the top of each figure

B Per cent surviving allografts in CBA mice infected with 500 irradiated *T spiralis* larvae (O--O) and in age-matched normal mice (●—●) Transplantation performed 1 week after infection.

other hand the allografts in mice with a curtailed infection were rejected earlier than those in mice with a normal infection.

Effect of infection with irradiated larvae on allograft survival time Infective larvae were sterilized by irradiation immediately before infection. Such larvae develop to adults but do not reproduce (9 17) Mice were

transplanted one week after infection with 500 irradiated larvae.



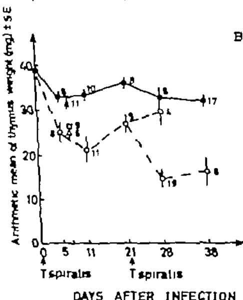


Fig. 4. Total body weight (A) and thymus weight (B) of CBA mice at various times after *T. spiralis* infection.
Mice infected with 500 larvae (O---O) mice infected with 500 larvae and challenged with the same dose 3 weeks later (O—O) and age-matched normal mice (●—●).
The weights determined one week after infection. Mice infected with 500 irradiated larvae (□) mice methyridine treated 4.5 days after infection (Δ) and methyridine treated normal mice (▲).
Number of animals recorded close to the symbols.

Fig. 3 B shows the mean survival time of the allografts in mice infected with irradiated larvae. It should be noted that the mean survival time in the latter was significantly longer than that in the controls ($p<0.01$). However the graft survival time in this group was shorter than in mice infected with non-irradiated larvae.

Recovery of muscle larvae from the diaphragms. All diaphragms were examined for muscle larvae and in all untreated mice infected with non-irradiated larvae parasites could be demonstrated.

No larvae were observed in the diaphragms of mice infected with irradiated larvae or treated with methyridine. The same result was obtained in uninfected mice.

Changes in body and thymus weights. Infected mice showed a steady loss in body weight which reached a maximum at about eleven days after infection. Thereafter they gradually gained weight but did not reach the levels observed in normal mice (Fig. 4 A). Mice given a second infection showed an additional and similar weight loss and recovery. However the body weight of infected methyridine treated mice and of mice infected with irradiated larvae did not differ from that of normal mice 7 days after infection. The mean weight of the thymus in *T. spiralis* infected animals decreased until day 11 ($p<0.001$) and then gradually increased to reach a normal level by day 28. Challenge was followed by a dramatic loss in thymus weight (Fig. 4 B). Also in infected, methyridine treated mice and in mice infected with irradiated larvae a significantly decreased weight of the thymus was recorded on day 7.

Histological examination of the thymus showed cortical depletion of lymphocytes. In some challenged mice cortex was almost completely devoid of thymocytes. There were no obvious changes in the medulla.

Adrenalectomized mice. Thymus weights of infected and uninfected mice were recorded 5 and 9 days after adrenalectomy. The results are shown in Fig. 5. There were no significant differences between the groups 5 days after

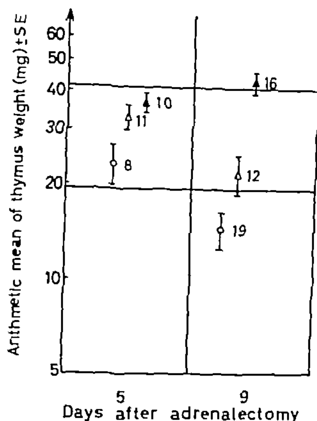


Fig 5 The thymus weight (arithmetic mean ± S E) in infected adrenalectomized CBA mice (Δ) in infected intact mice (○) and age-matched normal adrenalectomized mice (▲) The area within horizontal lines represents 95 per cent confidence limits for normal intact age-matched mice
The mice were infected with 500 *T spiralis* larvae and 3 weeks later challenged with 500 larvae Adrenalectomy performed 2 days before challenge. Number of animals recorded close to the symbols.

challenge (5 days after adrenalectomy) as compared with the normal mean thymus weight. Seven days after challenge, differences were significant adrenalectomy was followed by a significant increase in mean thymus weight in uninfected mice ($p < 0.001$) This did not occur in infected mice whose mean thymus weight was significantly below normal ($p < 0.05$) As before the mean weight of the thymus in infected but not adrenalectomized mice was very low ($p < 0.001$)

DISCUSSION

These intriguing results show that experimental trichinosis in mice is accompanied by depression of the humoral and the cellular immune responses to unrelated antigens. There is, however a clear-cut difference between the effect on the antibody response and the effect on the cell mediated response. The former is only depressed during a short period of the infection in contrast to the effect on the cell mediated response which is considerably more severe and longer lasting.

The results suggest that *T spiralis* infection depresses the T cell dependent antibody response but has little effect on, or actually increases, the antibody response to thymus independent antigens such as PVP The immunodepression is related to the stage of the infection. At one week when most parasites are in the intestine, there is no effect on either IgG or IgM responses to the injected sheep erythrocytes. However three weeks after infection, in the early stage of muscle infection there is a depression of the IgM response and this effect was greater in animals which had been challenged. A decrease in IgG response was also recorded 6 weeks after infection.

Taken together these observations suggest that *T spiralis* infection might impair T cell functions. This view is supported by the finding of cortical depletion of lymphocytes in the thymus which might lead to a reduction in the traffic of cortical lymphocytes to the medulla where maturation occurs. This could be expected to be reflected by a lowered output of immunocompetent cells resulting in depressed antibody responses to thymus dependent antigens.

The possibility that the thymic changes were due to corticosteroid effects was investigated. However the fact that the trichinella infection in adrenalectomized mice is still accompanied by thymic cell depletion rules out this hypothesis. It has also been shown that trichinosis is accompanied by only a slight increase in circulating corticosteroids (7)

The parallelism in loss of weight of the whole body and the thymus suggests that the thymic changes might be secondary effects of malnutrition However this is ruled out by the fact that there is still thymic atrophy in

mice receiving irradiated larvae or methyridine treatment and a loss in body weight is not seen in any of these situations.

Earlier work (12) has shown that new-born *T spiralis* larvae may release substance(s) which influence the plaque forming capacity of spleen lymphocytes to sheep erythrocytes. There is, however no evidence for their effect on cortical thymocyte depletion.

T spiralis infection seems to affect the T cell response at different levels. In addition to the moderate depression on antibody synthesis a profound and longlasting effect on the cell mediated immune response is demonstrated. This effect was most pronounced during the first week after infection when the parasite is found mainly in the host intestine. It also coincides with the development of specific cell mediated immunity (36).

Earlier studies have demonstrated immunity to challenge after infection with sterilized larvae (9 16). Immunity could also be shown when the primary infection was curtailed during the intestinal stage (10, 21). The present study shows that such procedures also lead to immunodepression, reflected as a delayed allograft rejection. Both specific immunity (9 10) and nonspecific immunodepression are, however less affected than in normal experimental infection. It is not clear by which mechanism *T spiralis* exerts its effect on allograft rejection. One possible explanation is that the larger antigenic load on the host organism occupies the cell mediated immune response to such an extent that the available capacity to reject allografts is significantly reduced.

This view is supported by a number of observations. As already mentioned, the period of strong immunodepression closely parallels the development of specific cell mediated immunity.

Secondly it has been shown that there is a proliferation of lymphoid cells during the intestinal stage both in Peyer's patches and in the intestinal mucosa (24 37). Such proliferation was not seen in T cell deprived mice (37) suggesting that, during this stage, there is a recruitment of large numbers of T cells to the intestine, which also normally contains a considerable fraction of the total pool of lymphocytes.

Simultaneously there is a significant depletion of the thymic cortex, but this finding cannot be directly associated with the prolonged survival time of allografts. Thus, in mice challenged 3 weeks after the primary infection, the thymic depletion was significantly stronger than that in primary infected mice while the immunodepressive effect was weaker. The latter finding might be due to the lower amount of antigen present in the intestine in challenged mice because of enhanced expulsion of adults.

Specific cell mediated immunity is less pronounced during the migration than during the intestinal stage (36). The present study shows that the same is true for depression of nonspecific cell mediated immunity. Instead, stimulation of specific antibody response was demonstrated (6, 26). The simultaneous production of circulating antibodies to *T spiralis* antigens and depression of the antibody response to unrelated thymus dependent antigens was demonstrated both in this study and by earlier workers (12, 27).

The migration stage is characterized by spread of larvae throughout the body mainly by vascular transport. During this period marked lymphoreticular reactions are seen reflected mainly as increase in size of the spleen and peripheral lymph nodes and, histologically as a considerable increase in the number of pyroninophilic cells both in thymus dependent and thymus independent areas of these organs (26). With the infective dose used, a great number of larvae should be produced, many of which could be expected to migrate later. The antigenic load therefore could be assumed to be heavy.

Although evidence has been presented to show that trichinosis is accompanied by nonspecific immunodepression which closely parallels the development of specific immunity the underlying mechanisms are not clear. Studies are in progress to investigate, at different times of *T spiralis* infection, the reactivity to various antigens and mitogens

of the cells participating in the immune responses.

We wish to thank Dr *Eva Gronowicz* Karolinska Institute, Sweden, for instructive information on haemolytic plaque assay and Dr *David Denham* London School of Hygiene and Tropical Medicine England for providing the methyridine The excellent technical assistance of *Maria Karpinska* and *Gunnel Lundgren* is gratefully acknowledged.

REFERENCES

1 *Andersson B* Induction of immunity and immunological paralysis in mice against poly vinyl pyrrolidone J Immunol *102* 1309–1313 1969

2. *Andersson B & Blomgren H* Evidence for thymus-independent humoral antibody producing in mice against polyvinyl pyrrolidone and *E. coli* lipopolysaccharide. Cellular Immunol. *2* 411–424 1971

3 *Barriga O O* Selective immunodepression in mice by *Trichinella spiralis* extracts and infections. Cellular Immunol. *17* 306–309 1975

4 *Bullock W W & Möller E* Spontaneous" B cell activation due to loss of a normal mouse serum suppressor Eur J Immunol. *2* 514–517 1972

5 *Campbell W C & Cuckler A C* Comparative studies on the chemotherapy of experimental trichinosis in mice Z. Tropenmed parasit *18* 408–417 1968

6. *Crandall R B & Crandall C A Trichinella spiralis* Immunologic response to infection in mice Exp Parasit. *31* 378–398 1972

7 *Cypess H R Lubiniecki A S & Hammon W McD* Immunosuppression and increased susceptibility to Japanese B Encephalitis Virus in *Trichinella spiralis* infected mice Proc Soc Exp Med Biol *143* 469–473 1973

8. *Denham D A* Studies with methyridine and *Trichinella spiralis* 1 Effect upon the intestinal phase in mice Expt. Parasit *17* 10–14 1965

9 *Denham D A* Immunity to *Trichinella spiralis* II Immunity produced by the adult worm in mice. Parasit *56* 745–751 1966

10 *Denham D A & Martinez A R* Studies with methyridine and *Trichinella spiralis*. 2 The use of the drug to study the rate of larval production in mice. J Helminth. *44* 357–363 1970

11 *Dresser D W & Wortis H H* Use of an antiglobulin serum to detect cells producing antibody with low haemolytic efficiency Nature (Lond.) *208* 859–861 1963

12 *Faubert G M* Depression of the plaque-forming cells to sheep red cells by the new born larvae of *Trichinella spiralis*. Immunol *30* 485–489 1976

13 *Faubert G & Tanner C E. Trichinella spiralis* Inhibition of sheep hemagglutinins in mice Exp. Parasit. *30* 120–123 1971

14 *Faubert G M & Tanner C E.* Leucoagglutination and cytotoxicity of the serum of infected mice and of extracts of *Trichinella spiralis* and the capacity of infected mouse sera to prolong skin allografts. Immunol. *28* 1041–1050 1975

15 *Goodwin L G Green D G Guy M W & Voller A* Immunosuppression during trypanosomiasis. Br J Exp. Path. *53* 40–43 1972.

16 *Gould S E. Gomberg H J Bethell F H Villella J B. & Hertz C S.*. Studies on *Trichinella spiralis* IV Effect of feeding irradiated Trichinella larvae on production of immunity to re-infection. Amer J Path. *31* 949–955 1955.

17 *Gould S E. Gomberg H J Villella, J B. & Hertz C S* Studies on *Trichinella spiralis*. VI Effect of cobalt-60 and X ray on morphology and reproduction. Amer J Path. *33* 79–105 1957

18. *Greenwood B M Playfair J H L & Torrigiani G* Immunosuppression in murine malaria. I General characteristics. Clin. exp. Immunol *8* 467–478 1971

19 *Huff R W Liebelt A K & Liebelt R. A.* Implantation of allogenic heart grafts in inbred mice. Cardiovasc Res. Center Bull. *6* 127–139 1968.

20 *Huldt G Gard S & Olovsson S G* Effect of *Toxoplasma gondii* on the thymus. Nature *244* 301 303 1973

21 *James E R & Denham D A* Immunity to *Trichinella spiralis* VI The specificity of the immune response stimulated by the intestinal stage J Helminth *49* 43–47 1975

22 *Jerne N K & Nordin A A* Plaque-formation in agar by single antibody producing cells. Science *140* 450 1963

23 *Judd K P & Trentin J J* Cardiac transplantation in mice I Factors influencing the take and survival of heterotopic grafts. Transpl *11* 298–302 1971

24 *Lark J E Jr & Race G J* A histopathologic study of the anterior small intestine of immunized and nonimmunized mice infected with *Trichinella spiralis* J Infect. Dis. *94* 262–272 1954

25 *Ljungström I* Antibody response to *Trichinella spiralis* In *Kim C W* (Ed.) Trichinellosis Proc 3rd Int. Conf on Trichinellosis.

Miami 1972. Intext Educational New York 1974.

26. *Ljungström I & Ruitenberg E. J.* A comparativ study of the immunohistological and serological response of intact and T cell-deprived mice to *Trichinella spiralis.* Clin. exp. Immunol. *24* 146–156, 1976.

27 *Lubiniecki A S & Cypess, R. H* Immunological sequelae of *Trichinella spiralis* infection in mice. Effect of the antibody responses to sheep erythrocytes and Japanese B Encephalitis Virus. Inf. Immun. *11* 1306–1311 1975

28 *Lubiniecki, A. S Cypess R. H & Lucas J P* Immune response to and distribution of sheep erythrocytes in *Trichinella spiralis* infected mice. Tropenmed. Parasit. *25* 345–349, 1974

29 *Phillips, R S., Selby G R. & Wakelin, D.* The effect of *Plasmodium berghei* and *Trypanosoma brucei* infections on the immune expulsion of the nematode *Trichuris muris* from mice. Int. J Parasit. *4* 409–415 1974

30 *Salaman M H Wedderburn N & Bruce-Chwatt L.* The immunodepressive effect of a murine plasmodium and its interaction with murine oncogenic viruses. J gen. microbiol. *59* 383–391 1969

31 *Schanzel, H & H gerat E.* The use of methyridine against the intestinal trichinosis in white mice. Acta universitatis agriculture, Brno, *12* 385–388, 1964

32. *Sterzl J & Riha, I* Detection of cells, producing 7 S antibodies by the plaque technique Nature (Lond.) *208* 858–859 1965.

33. *Strickland G T., Pettitt L. E. & Voller A.* Immunodepression in mice infected with *Toxoplasma gondii.* Amer J Trop. Med. Hyg. *22* 452–455 1973.

34 *S zkag, S E. & S killing, W:* Synergism between alloantibodies or F (ab') and prednisone in the survival of murine split heart allografts. Transpl. *15* 345–347 1973

35 *S et Moldavsky G J Shaghijan G. S Chernyakhovskaya I Y., Mkheidze D M., Litoshenko T A., Ozeretskovskaj N N & Kadaghidze Z. G* Inhibition of skin allograft rejection in Trichinella-infected mice Transpl. *9* 69–70 1970.

36. *Vernes A Floc'h, F., Biguet J & Tailliez R.:* Trichinose expérimentale – I. Cinétique des phénomènes d'hypersensibilité retardée chez la souris CBA et le rat Wistar Inter J Parasit. *5* 63–70 1975

37 *Walls, R. S Carter R. L., Leuchars E. & Davies A J S.:* The immunopathology of trichinosis in T-cell deficient mice. Clin. exp. Immunol. *13* 231–242, 1973.

Acta path microbiol. scand Sect. C, 85 142–148 1977

ANTIBODIES AGAINST *PSEUDOMONAS AERUGINOSA* IN SERUM FROM NORMAL PERSONS AND PATIENTS COLONIZED WITH MUCOID OR NON-MUCOID *PSEUDOMONAS AERUGINOSA* RESULTS OBTAINED BY CROSSED IMMUNOELECTROPHORESIS

NIELS HØIBY

Statens Seruminstitut, Department of Clinical Microbiology at Hvidovre Hospital, and the Paediatric Department TG Rigshospitalet, Copenhagen, Denmark

Høiby N Antibodies against *Pseudomonas aeruginosa* in serum from normal persons and patients colonized with mucoid or non mucoid *Pseudomonas aeruginosa* results obtained by crossed immunoelectrophoresis. Acta path. microbiol scand Sect. C, 85 142–148 1977

Serum from 154 normal persons was investigated for precipitating antibodies against *P aeruginosa* by crossed immunoelectrophoresis, using a polyvalent *P aeruginosa* standard antigen. Ninety four per cent of the sera contained no demonstrable precipitins, and 6 per cent contained precipitins against only one *P aeruginosa* antigen. Thirty-four per cent of sera from 122 patients colonized with *P aeruginosa* in various anatomical regions contained precipitins against 2 to 41 *P aeruginosa* antigens. Serum from 14 patients harbouring mucoid strains of *P aeruginosa* contained precipitins against these bacteria, and the average number of precipitins was significantly higher than the average number of precipitins in serum from patients harbouring non-mucoid strains The largest number of precipitins was found in serum from adult patients chronically colonized with mucoid *P aeruginosa* in the lower respiratory tract, and from adult patients with *P aeruginosa* bacteraemia originating from suppurative urinary tract infections caused by mucoid strains. Serum from patients with *P aeruginosa* cystitis or from patients with *P aeruginosa* colonization of the middle ear or with wound infection, contained only few or no precipitins.

Key words *P aeruginosa* antibodies immunoelectrophoresis.

N Høiby Department of Clinical Microbiology Hvidovre Hospital afs 445 Kettegaards Allé 30 DK 2650 Hvidovre Copenhagen Denmark

Received 27 ix.76 Accepted 30.xi 76

The humoral immune response against *Pseudomonas aeruginosa* has been investigated in cystic fibrosis patients by crossed immunoelectrophoresis. In cystic fibrosis patients chronically colonized by mucoid *P aeruginosa* strains, a large number of precipitins against these bacteria were found,

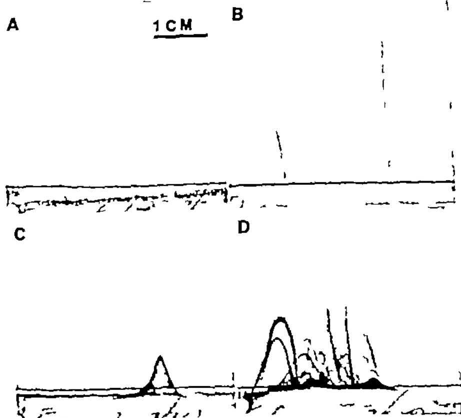


Fig. 1. Crossed immunoelectrophoresis of 2 µl *P. aeruginosa* antigen (St Ag) in the first dimension well against human serum (15 µl/cm²) in second dimension agarose. Technical: first dimension electrophoresis, anode to the right; second dimension electrophoresis, anode at the top. Staining: Coomassie brilliant blue.

A. Serum from 46-year-old normal female. One immunoprecipitate could be seen.

B. Serum from 95-year-old male with mucoid *P. aeruginosa* in the urine. Two immunoprecipitates could be seen.

C. Serum from 73-year-old tracheotomized woman with chronic bronchitis and non-mucoid *P. aeruginosa* in the tracheal secretion. Five immunoprecipitates could be seen.

D. Serum from 82-year-old male with pyonephrosis and bacteraemia caused by mucoid *P. aeruginosa*. Thirty-three immunoprecipitates could be seen.

whereas cystic fibrosis patients colonized with non-mucoid strains generally had only few or no precipitins against *P. aeruginosa* (10-12, 16).

This paper reports the results of investigation by crossed immunoelectrophoresis of the prevalence and number of *P. aeruginosa* precipitins in serum from normal persons and patients with diseases other than cystic fibrosis, who harboured mucoid or non-mucoid strains of *P. aeruginosa*.

MATERIAL AND METHODS

Normal Persons

Serum was taken from 154 'normal' persons (84 males, 70 females) without infections or immunological disorders. The age distribution is shown in Table 1. 151 of these persons had been

TABLE 1 *Pseudomonas aeruginosa Precipitins Revealed by Crossed Immunoelectrophoresis, in Healthy Persons of Different Ages*

Age in years (No. of persons)	0–15 (29)	16–29 (36)	30–49 (54)	50–69 (21)	70–91 (14)	Total (154)
0 precipitins	29	34	52	20	10	145
1 precipitin	0	2 (6 %)	2 (4 %)	1 (5 %)	4 (29 %)	9 (6 %)
(95 % c.l.)*	(0–12 %)	(1–19 %)	(0.5–13 %)	(0–24 %)	(8–58 %)	(3–11 %)

* c.l. confidence limits.

included in a previously published study concerning the more sensitive technique crossed immunoelectrophoresis with intermediate gel (14). None of the persons had a history of *P. aeruginosa* infection.

Patients

Serum was obtained from 122 patients (74 males, 48 females) harbouring *P. aeruginosa* in one or more anatomical regions on one or more occasions. The age distribution is shown in Fig. 2.

42 of the patients had *P. aeruginosa* in sputum or secretion obtained by endolaryngeal aspiration, and 33 of these patients had respiratory tract diseases, viz. chronic bronchitis, bronchiectasis and/or severe respiratory failure. Nine patients suffered from various other diseases—cardiac diseases, apoplexy and tetanus. In 29 cases the duration of the *P. aeruginosa* colonization was known (1 week to more than 2 years). Analysis of sweat electrolytes was performed on some of the patients whose symptoms might be mistaken for cystic fibrosis, but in no case could the diagnosis of cystic fibrosis be confirmed.

25 of the patients suffered from *P. aeruginosa* urinary tract infection, and 7 of these patients had an indwelling catheter. Duration of the infection was known in 22 of the cases (a few days to 4 months).

27 of the patients harboured *P. aeruginosa* in the middle ear and these patients suffered from chronic otitis media. 14 of these patients had previously undergone a middle ear operation. In 18 of these cases, the duration of the colonization was known (14 days to 7 years).

9 of the patients suffered from *P. aeruginosa* bacteraemia and 8 of these had also *P. aeruginosa* infection in another region (5 in the urinary tract, 1 in the respiratory tract, 1 in the gall-bladder and 1 case of meningitis). In 4 of the cases the duration of the latter infection was known (2 weeks to 6 months).

19 of the patients comprised a miscellaneous group. 15 of these harboured *P. aeruginosa* simultaneously in the respiratory tract and either the urinary tract or the middle ear and 4 harboured *P. aeruginosa* in wounds. Duration of the colonization was known in 17 of the cases (1 week to 1 year).

The serum was taken after *P. aeruginosa* had been isolated, identified and classified as mucoid or non-mucoid, according to previously published criteria (1, 10, 11, 15). The sera originated from hospitalized patients or out-patients treated at the Department of Infectious Diseases (including tracheotomized patients suffering from severe respiratory failure), the Department of Internal Medicine, the Department of Oto-rhino-laryngology, Blegdamshospitalet, and the Paediatric Clinic TG, Rigshospitalet, Copenhagen. The samples were stored at −30° C with NaN_3 added (15 mM/l).

Crossed Immunoelectrophoresis

This was performed by a microtechnique according to Weeke (1973) on 5 × 5 cm glass plates, as described previously (12). 2 µl of a polyvalent *P. aeruginosa* antigen preparation (St Ag) obtained by sonication (12) was run against patient serum (15 µl/cm²) in an agarose gel at pH 8.6. After staining with Coomassie brilliant blue Microme no. 1137 (E. Gurr Ltd., London) the number of different immunoprecipitates was counted. The number of precipitates has been shown to correlate with the titre of the strongest precipitins (10).

Statistical Methods

The Chi Square Test, the Fourfold Table Test, and the Mann-Whitney Rank Sum Test, were employed. Level of statistical significance: 5 per cent (double-tailed tests) (8).

RESULTS

Precipitating antibodies against one of the *P. aeruginosa* antigens were revealed in 9 (6 per cent) of the 154 normal sera (Fig. 1 A). These were from adult persons (Table 1).

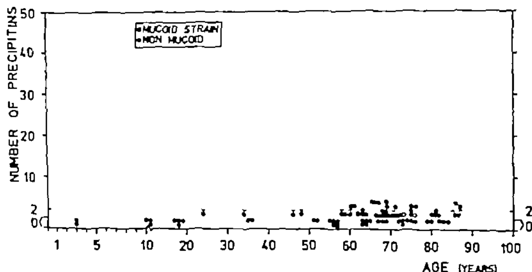


Fig 2 Number of precipitins against *P aeruginosa* in serum from 122 patients harbouring these bacteria plotted against the age of the patients The white dots represent patients harbouring mucoid strains of *P aeruginosa*, the black dots those with non-mucoid strains of *P aeruginosa* On the basis of the results in Table 1 *two or more precipitins are considered abnormal* (dotted line)

Of the 122 sera from patients, precipitating antibodies against *P aeruginosa* were found in 74 (61 per cent, 95 per cent confidence limits (c l) 52–69 per cent) A total of 349 precipitins were found in these 74 positive sera (mean 5 precipitins, range 1–41) Thirty two (26 per cent) of the sera contained only one precipitin against *P aeruginosa* and 42 (34 per cent) contained 2 or more precipitins (Fig 1 2 & 4)

Large numbers of *P aeruginosa* precipitins (≧10) were found in 7 adult patients only and only 2 of the children had 2 precipitins or more (Fig. 2)

As regards the 90 patients with known duration of the *P aeruginosa* colonization, more than 10 precipitins were found only in 3 patients who had been colonized for at least 6 months (Fig 3) However most (11/18) of the patients who had harboured *P aeruginosa* for at least 6 months had less than 2 precipitins (Fig. 3)

14 of the patients, representing most of the age groups (Fig 2) harboured mucoid strains of *P aeruginosa*. All sera from these patients contained *P aeruginosa* precipitins, and the prevalence of 2 or more precipitins was higher in serum from patients with mucoid strains (64 per cent) than in serum from patients with non-mucoid strains (31 per cent) ($p<0.025$) (Fig 2 & 4) The average number of *P aeruginosa* precipitins was also higher in the 14 sera from patients with mucoid strains (mean 12 precipitins) than in the positive sera from patients with non-mucoid strains (mean 3 precipitins ($p<0.05$) (Fig. 2 & 4)

The prevalence of sera with 2 or more precipitins was highest in patients with *P aeruginosa* bacteraemia (67 per cent) and lowest in patients harbouring these bacteria in the middle ear (22 per cent) ($p<0.05$) (Fig 4) Eight of the latter patients had harboured *P aeruginosa* in the middle ear for at least 6 months (Fig 3) Furthermore, the number of precipitins in sera with 2 or more precipitins was significantly higher in the groups of patients with *P aeruginosa* in the respiratory tract or in the blood than in patients with these bacteria only in the middle ear or in the urogenital tract ($p<0.01$) (Fig 4) The largest number of *P aeruginosa* precipi-

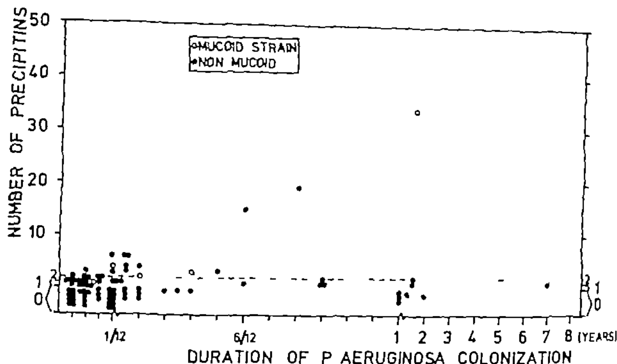


Fig 3 Number of precipitins against *P aeruginosa* in sera from 90 patients harbouring these bacteria, plotted against the duration of colonization. The duration of the colonization with *P aeruginosa* was unknown in 32 of the 122 patients shown in Fig. 2 & 4 and these patients are therefore not included in Fig 3 Black and white dots and dotted line as in Fig 2.

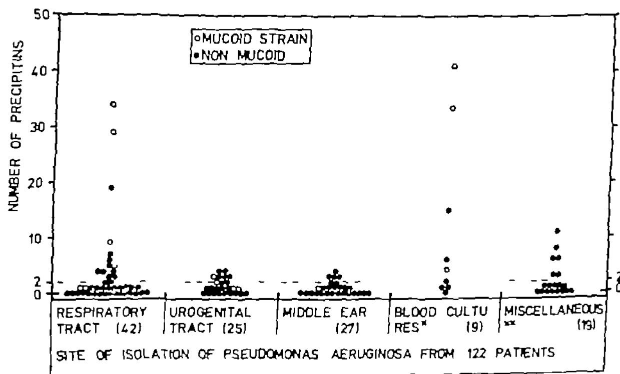


Fig 4 Number of precipitins against *P aeruginosa* in sera from 122 patients classified according to site of isolation of *P aeruginosa*. Black and white dots and dotted line as in Fig 2 Figures in brackets indicate the number of patients in each group x) 8 of these patients had *P aeruginosa* in another anatomical region besides the blood—for further details see Materials and Methods. xx) see Materials and Methods.

tins was found in sera from some of the patients with mucoid strains of these bacteria in the respiratory tract, or who suffered from *P aeruginosa* bacteraemia originating from suppurative infections of the urogenital tract (pyonephrosis, cystopyelitis + epididymal abscess)

DISCUSSION

Conventional agar-gel immunodiffusion techniques have not revealed *P aeruginosa* precipitins in normal unconcentrated human serum, although *P aeruginosa* antibodies could be demonstrated by agglutination or haemagglutination techniques (2-5 9 18, 19)

By means of the more sensitive crossed immunoelectrophoresis technique with intermediate gel, 54 per cent of the present normal sera were shown to contain one precipitin (no. 10 in the St Ag/St Ab reference system) and 4 per cent contained 2 precipitins (14) These precipitins cross-reacted with antigens present in a wide range of other bacterial species (14)

Using the present method, only 6 per cent of normal sera contained one demonstrable precipitin against *P aeruginosa* (corresponding to the cross-reactive antigen no. 10 (14)) The presence of two or more precipitins is indicative of a humoral immune response to *P aeruginosa* although there is a possibility of precipitins directed against cross-reactive antigens of other bacterial species, especially other Pseudomonas species (13 14) Although less sensitive the present modification of crossed immunoelectrophoresis is better adaptable to routine clinical work than crossed immunoelectrophoresis with intermediate gel, and the diagnostic sensitivity of the present method has proved satisfactory when working with serum from patients with cystic fibrosis and *P aeruginosa* infection (16)

Precipitating antibodies against *P aeruginosa* in serum from patients with *P aeruginosa* infection have been demonstrated previously by agar-gel immunodiffusion or conventional immunoelectrophoresis (2-6, 18, 19) and up to 8 precipitin bands have been found in patients sera by these methods. Moreover the development of precipitating antibodies and the number of precipitin bands have been found to correlate with the titre obtained by haemagglutination methods (5-7 18, 19) The present results are in agreement with other studies which show that one-half to two-thirds of patients colonized with *P aeruginosa* have demonstrable precipitins against these bacteria in their serum (3-5 18) However the number of precipitins found in some of the present patients—notably some of those chronically harbouring *P aeruginosa*—was up to 5 times greater than that reported by authors using conventional methods (2-6 18, 19) This is in accordance with the results obtained previously when the present methods were used on sera from patients with cystic fibrosis (12, 16)

As in previous studies of patients with cystic fibrosis, colonization with mucoid strains of *P aeruginosa* was associated with a higher prevalence and a greater number of precipitins than non-mucoid strains. In some of the adult patients, the number of precipitins was even comparable with the findings in children with cystic fibrosis (3 12, 16)

The nature of the mechanism favouring the unstable mucoid strains which are so frequent in cystic fibrosis patients is unknown, but several possible factors, including the humoral immune response have been discussed elsewhere (1 10-12) The present findings are not contrary to the view that the humoral immune response may favour the survival of mucoid strains as compared to non-mucoid strains, since the former are more resistant to the defence mechanisms of the immunized host (1 10-12)

The present study shows that crossed immunoelectrophoresis can be used to reveal a humoral immune response against *P aeruginosa* in serum from some of the patients harbouring these bacteria. The information obtained by this method has proved to be of diagnostic value in studies of cystic fibro-

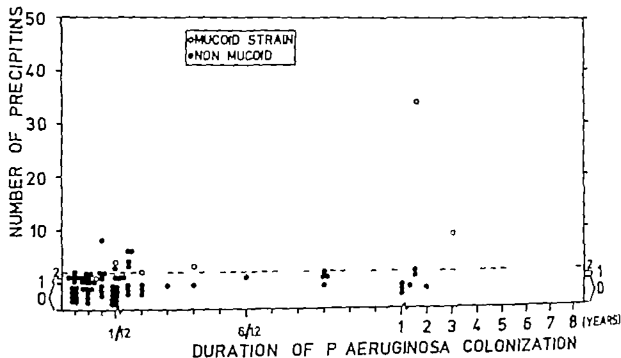


Fig 3 Number of precipitins against *P aeruginosa* in sera from 90 patients harbouring these bacteria, plotted against the duration of colonization. The duration of the colonization with *P aeruginosa* was unknown in 32 of the 122 patients shown in Fig 2 & 4 and these patients are therefore not included in Fig 3 Black and white dots and dotted line as in Fig 2.

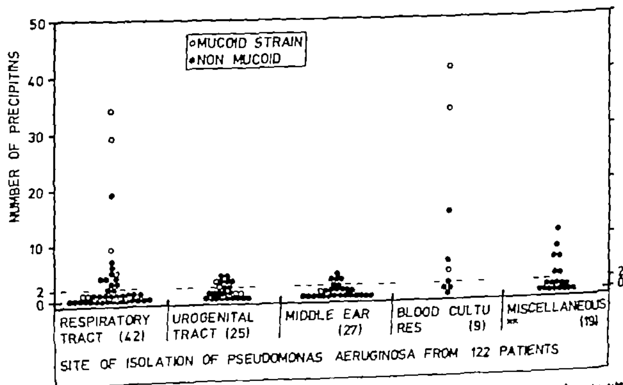


Fig 4 Number of precipitins against *P aeruginosa* in sera from 122 patients classified according to site of isolation of *P aeruginosa* Black and white dots and dotted line as in Fig 2. Figures in brackets indicate the number of patients in each group x) 8 of these patients had *P aeruginosa* in another anatomical region besides the blood—for further details see Materials and Methods xx) see Materials and Methods.

Acta path. microbiol. scand. Sect. C, 85 149–152, 1977

PSEUDOMONAS AERUGINOSA INFECTION IN CYSTIC FIBROSIS

Relative Prevalence of Antibodies in Serum against Cathodic and Anodic Migrating P aeruginosa Antigens Determined by Immunoelectrophoretic Methods

Niels Høiby

Statens Seruminstitut, Department of Clinical Microbiology at Hvidovre Hospital, and the Paediatric Department TG, Rigshospitalet, Copenhagen, Denmark

Høiby N *Pseudomonas aeruginosa* infection in cystic fibrosis. Relative prevalence of antibodies against cathodic and anodic migrating antigens determined by immunoelectrophoretic methods. Acta path. microbiol. scand. Sect. C, 85 149–152 1977

Sera from 119 patients with cystic fibrosis were examined for precipitating antibodies against anodic migrating *P aeruginosa* antigens by means of crossed immunoelectrophoresis. The presence of precipitins against cathodic migrating *P aeruginosa* antigens was investigated by means of classical immunoelectrophoresis. Thirty-six per cent of the sera contained precipitins against both anodic and cathodic antigens, 20 per cent contained only precipitins against anodic antigens, none contained only precipitins against cathodic antigens, and 44 per cent had no demonstrable *P aeruginosa* precipitins. The amount of extra information obtained by combining the results of classical immunoelectrophoresis with the results of crossed immunoelectrophoresis was small

Key words Cystic fibrosis; immunoelectrophoresis *Pseudomonas aeruginosa*.

N. Høiby Department of Clinical Microbiology H idovre Hospital afs. 445, Kettegaards Allé 30, DK 2650 H idovre Copenhagen, Denmark.

Received 27 ix.76 Accepted 30.xi.76

Quantitative immunoelectrophoretic methods have revealed 64 different anodic migrating antigens in *P aeruginosa* (8–11) and classical immunoelectrophoresis according to *Grabar & Williams* (1953) has demonstrated 5 cathodic migrating *P aeruginosa* antigens at pH 8.6 using the same antigen-antibody system The migration of the cathodic antigens can be changed by carbamylation (*Weeke* 1973) but some of the antigens of the standard *P aeruginosa* antigen preparation (St-Ag) are destroyed by this procedure (disappearance of immunoprecipitates when tested with a standard *P aeruginosa* antiserum (St-Ab) unpublished results)

By means of crossed immunoelectrophoresis, it has been shown that patients with cystic fibrosis (CF) suffering from chronic infection with mucoid *P aeruginosa* produce antibodies against many of the antigens of these bacteria (7 12) However precipitins against cathodic migrating antigens (pH 8.6) are not detected by that method.

The aim of the present study was to investigate the relative prevalence of precipitating antibodies in CF sera against anodic and cathodic migrating *P aeruginosa* antigens by

sus patients (16) The present results indicate that superficial colonization with *P aerugi nosa* (*e g* of the middle ear) gives rise to only a few or no precipitins, whereas suppurative infections with these bacteria (*e.g* of the lower respiratory tract or the upper urinary tract) give rise to many precipitins.

This work was aided by grants from the *Thorvald Madsen Legat* the *Danish Medical Research Council* the *National Danish Association Against Cystic Fibrosis* and the *Family Hede Nielsens Foundation*. Thanks are due to *Anni Bethsen Ase Meyer* and *Birgitte Skjolstrup* for skillful technical assistance

REFERENCES

1 *Bergan T & Høiby N* Epidemiological markers for *Pseudomonas aeruginosa* 6 Relationship between concomitant non-mucoid and mucoid strains from the respiratory tract in cystic fibrosis Acta path. microbiol. scand. Sect. B *83* 553–560 1975

2 *Burns M W* The significance of various bacteria in chronic bronchial disorders as determined by bacterial antibody detection. Aust. Ann. Med *17* 289–294 1968.

3 *Burns M W & May J R* Bacterial precipitins in serum of patients with cystic fibrosis. Lancet *i* 270–272 1968

4 *Crowder J G & White A* A serologic response in human Pseudomonas infection. J Lab Clin. Med *75* 128–136 1970

5 *Crowder J G Fisher M W & White A* Type-specific immunity in pseudomonas diseases J Lab. Clin. Med. *79* 47–54 1972.

6 *Crowder J G Devlin H B Fisher M & White A* Heatstable opsonins in sera of patients with *Pseudomonas* infection. J Lab Clin. Med *83* 853–859 1974

7 *Diaz F & Neter E. Pseudomonas aeruginosa* Serogroups and antibody response in patients with neoplastic diseases. Am. J Med. Sci. *259* 340–345 1970

8. *Diem K* (Ed) *Documenta Geigy Scientific Tables*. J A. Geigy S. A. Basle 1962, pp. 36 109–123 & 124–127

9 *Garnes S & Landy M*. Prevalence of antibody to Pseudomonas in normal human sera. J Bact. *69* 628–633 1955

10 *Høiby N & Axelsen N H* Identification and quantitation of precipitins against *Pseudomonas aeruginosa* in patients with cystic fibrosis by means of crossed immunoelectrophoresis with intermediate gel. Acta path. microbiol. scand. Sect. B, *81* 298–308, 1973

11 *Høiby N* Epidemiological investigations of the respiratory tract bacteriology in patients with cystic fibrosis. Acta path. microbiol. scand. Sect. B, *82* 541–550, 1974

12. *Høiby N Pseudomonas aeruginosa* infection in cystic fibrosis. Relationship between mucoid strains of *Pseudomonas aeruginosa* and the humoral immune response Acta path. microbiol. scand. Sect. B, *82* 551–558, 1974

13 *Høiby N* Cross-reactions between *Pseudomonas aeruginosa* and thirty-six other bacterial species. Scand. J Immunol. *4* Suppl. 2 187–196 1975

14 *Høiby N* Normally occurring precipitating antibodies against *Pseudomonas aeruginosa*. Prevalence, specificities and titres. Scand. J Immunol. *4* Suppl. 2 197–202 1975

15 *Høiby N* Prevalence of mucoid strains of *Pseudomonas aeruginosa* in bacteriological specimens from patients with cystic fibrosis and patients with other diseases. Acta path. microbiol scand. Sect. B, *83* 549–552, 1975.

16 *Høiby N Flensborg, E W Beck B. Jacobsen S V & Jacobsen L. Pseudomonas aeruginosa* infection in cystic fibrosis. Diagnostical and prognostical significance of *Pseudomonas aeruginosa* precipitins determined by means of crossed immunoelectrophoresis. In press Scand. J resp. Dis. 1977

17 *Weeke B.* Crossed immunoelectrophoresis Scand. J Immunol. *2* Suppl. 1 47–56 1973.

18 *Young L S Yu B H & Armstrong, D* Agar-gel precipitating antibody in *Pseudomonas aeruginosa* infections. Infect. and Immun. *2* 495–503 1970

19 *Young, L. S* Role of antibody in infections due to *Pseudomonas aeruginosa*. J Infect. Dis. *130 Suppl.* 111–118 1974

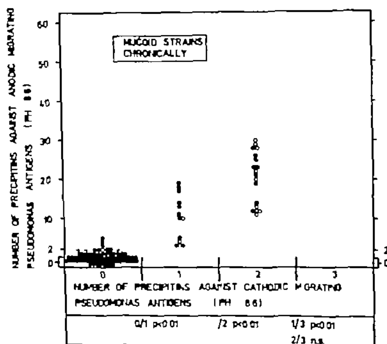


Fig. 2. The number of precipitins against anodic and cathodic migrating *P. aeruginosa* antigens is shown by dots, each of which represents serum from one of the 119 cystic fibrosis patients. Precipitins against anodic antigens were detected by crossed immunoelectrophoresis and precipitins against cathodic antigens by classical immunoelectrophoresis. Two or more precipitins against anodic antigens are considered to be indicative of humoral immune response against *P. aeruginosa* (dotted line) (13). White dots represent the 45 patients who harboured mucoid or alternating mucoid and non-mucoid *P. aeruginosa* chronically. Black dots represent the remaining 74 patients. The number of precipitins against anodic antigens in sera with 0 vs. 1 etc. precipitins against cathodic antigens was compared by the Mann-Whitney Test (double tailed) (4) and the p-values are given in the figure. n.s. = not significant (p>5 per cent).

The number of precipitins against anodic antigens increased the greater the number of precipitins against cathodic antigens (Fig. 2). Moreover all the patients with precipitins against 2 or 3 cathodic antigens harboured mucoid or alternating mucoid and non-mucoid strains of *P. aeruginosa* chronically (Fig. 2).

Classical immunoelectrophoresis has been used by other authors to detect *P. aeruginosa* precipitins in sera from CF patients (2, 3, 5, 14). Some of these authors (2) found precipitins against up to 5 *P. aeruginosa* antigens, two of which migrated cathodically at pH 8.2. In comparison with those studies, an increased amount of information has been obtained concerning the antibody response to *P. aeruginosa* antigens by means of crossed immunoelectrophoresis and the methods used in the present work for antigen production. This is in agreement with experience from other microbial antigen-antibody systems (1). The present study does not indicate that the diagnostic and prognostic significance of results obtained by crossed immunoelectrophoresis (12) would be altered by an additional evaluation of precipitins against cathodic migrating antigens by the classical immunoelectrophoresis method.

This work was aided by a grant from the *Family Hede Nielsens Foundation*. The author is grateful to Mrs. *Anni Bethien* for skilful technical assistance.

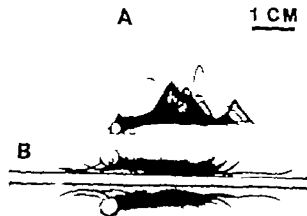


Fig 1 A Crossed immunoelectrophoresis of 2 µl *P aeruginosa* antigen (St-Ag) in the well against serum (15 µl/cm²) from a cystic fibrosis patient suffering from a chronic lung infection with mucoid *P aeruginosa*. First dimension electrophoresis anode to the right second dimension electrophoresis anode at the top Sixty immunoprecipitates *could be counted on this plate.*

B Classical immunoelectrophoresis of 10 µl *P aeruginosa* antigen (St-Ag) in the wells against 200 µl of the same serum as used in *A* in the trough anode to the right Three cathodic immunoprecipitates could be counted on this plate The anodic immunoprecipitates correspond to the 60 precipitates which could be counted in *A* but the resolution of the precipitate pattern is inferior to that obtained in *A* (only 17 precipitates could be *distinguished in B*)

comparing results obtained by crossed immunoelectrophoresis with those obtained by classical immunoelectrophoresis.

MATERIALS AND METHODS

Patients and normal persons Serum was obtained from 119 CF patients (67 males and 52 females aged 4–28 years) attending the CF Clinic TG Rigshospitalet, Copenhagen Forty five of the patients harboured mucoid or alternating mucoid and non-mucoid strains of *P aeruginosa* chronically in the lower respiratory tract 3 patients harboured non mucoid *P aeruginosa* chronically 10 patients harboured these bacteria intermittently and 61 patients had, *to our knowledge never harboured P aeruginosa* in the respiratory tract. Furthermore sera were obtained from 29 of the normal persons (12 males, 17 females aged 2–64 years) described previously (13)

Immunoelectrophoretic methods Serum was examined for precipitating antibodies against anodic migrating antigens of *P aeruginosa* by means of crossed immunoelectrophoresis on 5 x 5 cm glass plates (7 10) Two µl of a polyvalent *P aeruginosa* antigen (St Ag) (9) was run against patient serum (15 µl/cm²) in an agarose gel (Indubiose A 37 Batch FF3968 L'Industrie Biologique Française S.A.) at pH 8 6 (tris-barbital buffer ionic strength 0.02) Serum was investigated for precipitating antibodies against cathodic migrating *P aeruginosa* antigens by classical immunoelectrophoresis (6) Ten µl St Ag was separated electrophoretically in an agarose gel using the same conditions *as for the first dimension electrophoresis of* crossed immunoelectrophoresis (7 10) Following electrophoresis, diffusion took place against 200 µl serum from the patient at 4 C in a humid atmosphere for 48 h. The gels were washed, and the immunoprecipitates stained with Coomassie brilliant blue

RESULTS AND DISCUSSION

Two (7 per cent) of the 29 normal sera had detectable precipitins against only one anodic migrating *P aeruginosa* antigen which was detected by both crossed immunoelectrophoresis and classical immunoelectrophoresis. None of the normal sera contained detectable precipitins against cathodic migrating antigens.

Forthy three of the 119 CF patients (36 per cent) had demonstrable precipitins in serum against both anodic and cathodic *P aeruginosa* antigens, 24 (20 per cent) patients had only precipitins against anodic antigens, *none of the patients had only precipitins* against cathodic antigens, and 52 (44 per cent) had no demonstrable precipitins against these bacteria (*Fig 1 & 2*)

Classical immunoelectrophoresis revealed no precipitins against anodic *P aeruginosa* antigens in sera which were negative in crossed immunoelectrophoresis.

Altogether 1020 precipitins were counted in the 67 positive sera 944 (92.5 per cent) of these reacted with anodic *P aeruginosa* antigens and 76 (7.5 per cent) reacted with cathodic antigens (Fig 1 & 2)

Up to 60 precipitins against anodic antigens and up to 3 precipitins against cathodic antigens were counted in one serum (Fig 1 & 2)

Acta path. microbiol. scand. Sect. C, 85 153–160, 1977

STRUCTURAL SIMILARITIES BETWEEN A PROTEIN EXTRACTED FROM NORMAL HUMAN TISSUES AND A COMPONENT OF AMYLOID FIBRILS

GUNNAR HUSBY and KNUT SLETTEN

Institute of Immunology and Rheumatology, Rikshospitalet and Institute of Biochemistry, University of Oslo, Norway

Husby G & Sletten, K. Structural similarities between a protein extracted from normal human tissues and a component of amyloid fibrils. Acta path. microbiol. scand. Sect. C, 85 153–160, 1977

Comparative immunologic and chemical studies of crude or fractionated amyloid fibrils and materials obtained from corresponding normal tissues have been performed. Materials from the amyloidotic and normal tissues were subjected to identical methods of extraction, chemical treatment and protein fractionation. Antigenic similarities between crude amyloid fibrils and corresponding normal tissue extracts were observed, however, a considerably larger concentration of the latter antigens was needed to obtain immunologic reactivity in double diffusion in gel. Striking similarities were observed when the amino acid composition of a high molecular weight subcomponent of amyloid fibrils was compared with that of normal tissue extracts. Crude, intact amyloid fibrils were highly effective in absorbing Congo-red while the ability to absorb Congo-red was by far less if the high molecular weight subcomponent of amyloid fibrils as well as the corresponding normal tissue extracts were used. The high molecular weight subcomponent of amyloid, which seems to be an integral part of the amyloid fibrils, most probably is a protein derived from normal tissue.

Key words: Amyloid fibrils; normal human tissues; protein extraction; structural similarities.

G. Husby, Institute of Immunology and Rheumatology, Fredrikke Qvams gt. 1, Oslo 1, Norway

Received 13.ii.76   Accepted 15.iii.76

It was shown by *Pras et al.* (21, 22) that amyloid fibrils can form a stable suspension in distilled water, while the presence of even minor amounts of salt makes the fibrils precipitate very rapidly. Amyloid fibrils can therefore be purified from amyloid-laden organs by first washing homogenized tissue several times in physiological saline to remove all soluble proteins, and thereafter extracting the fibrils with distilled water. This procedure has become the standard method for isolation of amyloid fibrils (10). After extraction, the amyloid fibrils can be solubilized by treatment with alkali (22), acid (20), guanidine (9) or urea (4), and thereafter fractionated by gel filtration under dissociating conditions (9). The subsequent immunologic and chemical studies have given evidence of different protein subunits of amyloid fibrils. Two major classes of amyloid proteins have been characterized. One is of homogenous im-

REFERENCES

1. *Axelsen N H* Analysis of human Candida precipitins by quantitative immunoelectrophoresis A model for analysis of complex microbial antigen antibody systems. Scand. J Immunol. *5* 177–190 1976
2. *Burns M W & May J R* Bacterial precipitins in serum of patients with cystic fibrosis. Lancet *i* 270–272 1968.
3. *Burns M W* The pattern of bacterial infection in bronchial diseases in Australia. A serological and bacteriological survey Med. J Aust. *2* 697–701 1972
4. *Diem K* (Ed) Documenta Geigy Scientific Tables. J R. Geigy S.A., Basel 1962 p 125–127
5. *Doggett R G & Harrison G M* *Pseudomonas aeruginosa* Immune status in patients with cystic fibrosis. Infect. and Immun. *6* 628–635 1972.
6. *Grabar P & Williams C.A* Methode permettant l'étude conjugée des propriétetés électrophorétiques et immunochimiques d un mélange de proteines application au sérum sanguin. Biochim. biophys. Acta *10* 193–194 1953
7. *Høiby N* *Pseudomonas aeruginosa* infection in cystic fibrosis. Relationship between mucoid strains of *Pseudomonas aeruginosa* and the humoral immune response. Acta path. microbiol scand. Sect B *82* 551–558 1974
8. *Høiby N* The serology of *Pseudomonas aeruginosa* analysed by means of quantitative immunoelectrophoretic methods. I Comparison of thirteen O groups of *Ps. aeruginosa* with a polyvalent *Ps aeruginosa* antigen-antibody reference system. Acta path. microbiol. scand. Sect. B, *83* 321–327 1975
9. *Høiby N.* The serology of *Pseudomonas aeruginosa* analysed by means of quantitative immunoelectrophoretic methods. III Reproducibility of a polyvalent *P aeruginosa* reference standard-antigen. Acta path. microbiol. scand. Sect. B, *83* 433–442, 1975
10. *Høiby N* Cross-reactions between *Pseudomonas aeruginosa* and thirty-six other bacterial species. Scand. J Immunol *4* Suppl. 2, 187–196 1975
11. *Høiby N* The serology of *Pseudomonas aeruginosa* analysed by means of quantitative immunoelectrophoretic methods. IV Production of polyvalent pools of rabbit antiserum against *P aeruginosa* (reference standard antibody) Acta path microbiol Sect. C, *84* 372–381, 1976.
12. *Høiby N Flensborg E. W., Beck B., Friis, B Væder Jacobsen S & Jacobsen L.* *Pseudomonas aeruginosa* infection in cystic fibrosis Diagnostic and prognostic significance of *Pseudomonas aeruginosa* precipitins determined by means of crossed immunoelectrophoresis. In press. Scand. J resp Dis 1977
13. *Høiby N* Antibodies against *Pseudomonas aeruginosa* in serum from normal persons and patients colonized with mucoid or non-mucoid *Pseudomonas aeruginosa*. results obtained by crossed immunoelectrophoresis. Acta path. microbiol scand Sect. C, *85* 142–148, 1977
14. *May J R Herrick N C & Thompson D* Bacterial infection in cystic fibrosis. Arch Dis. Childh. *47* 908–913 1972
15. *Weeke B* General remarks on principles, equipment, reagents and procedures. Scand. J Immunol. *2* Suppl. 1 15–33 1973 a.

accident (Table 1). The organs were obtained within 4 hours of death and kept frozen at −20° C until used. The normal tissues were subjected to exactly the same extraction, solubilization and gel filtration procedures as described for corresponding amyloid-laden organs.

Immunologic studies. Double immunodiffusion in 1 per cent agarose gel with barbital buffer pH 8.6 was used for antigenic characterization of alkali- or guanidine-treated amyloid fibrils and normal tissue preparations (11, 15). Materials obtained by gel filtration were also subjected to immunodiffusion experiments (15). Antisera to the following protein antigens were employed: Human plasma and various normal serum protein including immunoglobulins and their κ and λ light chains, amyloid protein AA (16), amyloid protein AR (VλV 13), amyloid P-component (5) kindly provided by dr. *A. S. Cohen* and finally the amyloid V_0-material (13, 15).

Amino acid analyses. Amino acid composition and partial N-terminal sequence studies were performed as previously described (15, 24, 25).

Congo-red absorption. The method established by *Prus et al.* (21) was used for Congo-red absorption experiments. Briefly, 2.5 mg of lyophilized amyloid fibrils and corresponding normal tissue extracts were suspended by sonication in 1 ml of distilled water and mixed with 5 ml of a 0.1 mg/ml solution of Congo-red in physiological saline, incubated at 4° C for 2 hours under constant stirring and thereafter centrifuged at 1000 × g for 10 minutes. The supernatant was read at 490 nm in a spectrophotometer. The sediments were kept for polarization microscopy (11) after two washes in physiological saline.

RESULTS

Yield of water-extractable material from amyloid-laden and normal tissues. The dry weight of lyophilized water extracts from 20 gram of fresh or frozen/thawed amyloid-laden and corresponding normal human organs is shown in Table 1. The yield obtained from the amyloid-laden organs was consistently much higher than that obtained from normal tissue. However, a considerable amount of water-extractable material was also obtained from the normal organs, thus permitting further comparative studies of normal and amyloid rich tissues. No normal plasma proteins could be detected by immunodiffusion tests in the water-extracted materials from amyloidotic or normal organs.

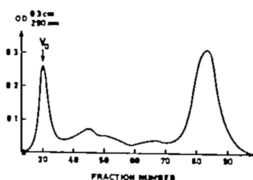


Fig. 1 a. Gel filtration on 3.2 × 96.5 cm Sephadex G-100 column with V_0 = 157 ml equilibrated with 5 M guanidine-HCl in 1 N acetic acid of amyloid fibrils (J.B. liver) treated with 0.55 M Tris-HCl buffer pH 8.5 containing 6 M guanidine-HCl and 0.1 M dithio-threitol. Fraction volume 5.4 ml. Fractions of the first peak (V_0) represent the void volume material (19 per cent of the proteins eluted) while the major retarded peak corresponding to fractions no. 78–90 represent the amyloid protein AA eluted with a yield of 58 per cent.

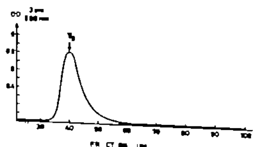


Fig. 1 b. Gel filtration on a 3.2 × 96.5 cm. Sephadex G-100 column with V_0 = 157 ml equilibrated with 5 M guanidine HCl in 1 N acetic acid of water-extracted protein from normal human liver treated with 0.55 M Tris-HCl buffer pH 8.5 containing 6 M guanidine-HCl and 0.1 M dithiothreitol. Fraction volume 3.9 ml. Practically all the protein material is eluted in the void volume.

Gel filtration patterns. A typical example of Sephadex G-100 gel filtration profile of guanidine dithiothreitol-treated amyloid fibrils of the protein AA type is shown in Fig. 1 a. Two major protein components were obtained: one was excluded by the column and hence eluted in the void volume (V_0-material). The other component protein AA, was considerably retarded, corresponding to a

11*

TABLE 1 *Source of Tissues and Yield of Water-extractable Materials*

Source of tissue	Diagnosis	Organ	Yield of material extracted with water* mg	Yield of V_0-material§ %
J.L.	Ankylosing spondylitis/ amyloidosis	Liver	1 100	20
J.L.	Ankylosing spondylitis/ amyloidosis	Spleen	900	21
T.H.	Juvenile rheumatoid arthritis/amyloidosis	Liver	500	38
J.B.	Waldenström's macro-globulinaemia/amyloidosis	Liver	700	19
A.R.	Primary amyloidosis	Spleen	1,000	25
N.N.	Normal Control	Liver	310	95
N.N.	Normal Control	Spleen	250	92

* Starting material 20 grams of fresh tissue.

§ Determined by estimation of the area of the protein peaks monitored at 280 nm. The absorptions at 280 nm were recorded on a logaritmic scale and areas of the peaks determined after conversion of the logaritmic recording to a linear one.

munoglobulin light chain nature (8) and is connected mainly with primary or myeloma associated amyloidosis. The other subunit, the amyloid protein AA (for nomenclature, see reference 2) is of unknown origin (3 4 6 15 16 17 24) and is found at high concentration particularly but not exclusively in secondary amyloidosis (3 16) and in experimental amyloidosis in various animals (7 14 23). In addition, a material of higher molecular weight, the void volume or "V_0-material" is also consistently eluted during gel filtration of solubilized amyloid fibrils (3 11 13 17). It was the aim of this study to compare the amyloid V_0 material and a protein component prepared from corresponding normal organs using the same procedure for extraction.

MATERIALS AND METHODS

Amyloid fibril preparations Amyloid fibrils were prepared from the liver of a patient (T.H.) with juvenile rheumatoid arthritis, from the spleen and liver of a patient (J.L.) with amyloidosis secondary to ankylosing spondylitis, and from the liver of a patient (J.B.) with amyloidosis associated with Waldenström's macroglobulinemia (Table 1). The amyloid fibrils from these patients have previously been shown to be of the protein AA type (15, 16, 24). Amyloid fibrils were also extracted from the spleen of a patient with primary amyloidosis of the immunoglobulin type (proteins AR, V λ V) previously described (15 25) (Table 1).

The amyloid fibrils were isolated according to *Pras et al.* (21 22) using extractions with distilled water after repeated washings of homogenized tissue with physiological saline until the optical density at 280 nm in the supernatant was negligible (11). The first distilled water supernatant was discarded while supernatants no 2 3, 4 5 and 6 containing the extracted amyloid fibrils were lyophilized and kept at −20 C until used. For the preparation of high molecular weight V_0-material lyophilized amyloid fibrils were dissolved (20 mg/ml) in 6M guanidine/0.1 M dithiothreitol (DTT) and gel filtered on a Sephadex G-100 column equilibrated with 5M guanidine/1N acetic acid (15). Protein excluded by this column and thus eluted in the void volume (the V_0 material) was collected dialysed against distilled water and lyophilized. Amyloid fibrils were also treated with 0.1 N NaOH (2 mg/ml) for 2 hours and thereafter dialyzed against distilled water. The resulting alkali-degraded amyloid fibrils (DAM)* were used for immunization of rabbits to produce anti-amyloid antiserum, and for immunologic studies (11).

Normal tissue preparations Normal spleen and liver tissues were obtained from a 32 years old previously healthy female (N.N.) who died in a traffic

* DAM = degraded amyloid fibrils.

accident (Table 1) The organs were obtained within 4 hours of death and kept frozen at −20° C until used. The normal tissues were subjected to exactly the same extraction, solubilization and gel filtration procedures as described for corresponding amyloidladen organs.

Immunologic studies. Double immunodiffusion in 1 per cent agarose gel with barbital buffer pH 8.6 was used for antigenic characterization of alkali- or guanidine-treated amyloid fibrils and normal tissue preparations (11 15) Materials obtained by gel filtration were also subjected to immunodiffusion experiments (15) Antisera to the following protein antigens were employed Human plasma and various normal serum protein including immunoglobulins and their κ and λ light chains amyloid protein AA (16) amyloid protein AR (VλV 13) amyloid P-component (5) kindly provided by dr *A S Cohen* and finally the amyloid V_0-material (13 15)

Amino acid analyses. Amino acid composition and partial N-terminal sequence studies were performed as previously described (13 24 25)

Congo-red absorption. The method established by *Pras et al* (21) was used for Congo-red absorption experiments. Briefly 2.5 mg of lyophilized amyloid fibrils and corresponding normal tissue extracts were suspended by sonication in 1 ml of distilled water and mixed with 5 ml of a 0 1 mg/ml solution of Congo-red in physiological saline Incubated at 4 C for 2 hours under constant stirring and thereafter centrifuged at 1000 × g for 10 minutes. The supernatant was read at 490 nm in a spectrophotometer The sediments were kept for polarization microscopy (11) after two washes in physiological saline

RESULTS

Yield of water-extractable material from amyloid-laden and normal tissues The dry weight of lyophilized water extracts from 20 gram of fresh or frozen/thawed amyloid laden and corresponding normal human organs is shown in Table 1 The yield obtained from the amyloid laden organs was consistently much higher than that obtained from normal tissue However a considerable amount of water-extractable material was also obtained from the normal organs, thus permitting further comparative studies of normal and amyloid rich tissues No normal plasma proteins could be detected by immunodiffusion tests in the water-extracted materials from amyloidotic or normal organs.

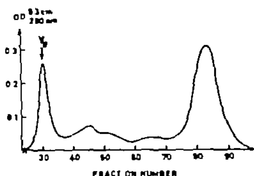


Fig 1 a. Gel filtration on a 3.2 × 96.5 cm Sephadex G-100 column with V_0 = 157 ml equilibrated with 5 M guanidine-HCl in 1 N acetic acid of amyloid fibrils (J B., liver) treated with 0.55 M Tris-HCl buffer pH 8 5 containing 6 M guanidine-HCl and 0.1 M dithio-threitol. Fraction volume 5 4 ml Fractions of the first peak (V_0) represent the void volume material (19 per cent of the proteins eluted) while the major retarded peak corresponding to fractions no. 78-90 represent the amyloid protein AA eluted with a yield of 58 per cent

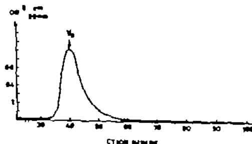


Fig 1 b Gel filtration on a 3.2 × 96.5 cm. Sephadex G-100 column with V_0 = 157 ml equilibrated with 5 M guanidine-HCl in 1 N acetic acid of water-extracted protein from normal human liver treated with 0.55 M Tris-HCl buffer pH 8.5 containing 6 M guanidine-HCl and 0 1 M dithiothreitol. Fraction volume 3.9 ml. Practically all the protein material is eluted in the void volume.

Gel filtration patterns A typical example of Sephadex G-100 gel filtration profile of guanidine dithiothreitol-treated amyloid fibrils of the protein AA type is shown in Fig. 1 a. Two major protein components were obtained one was excluded by the column and hence eluted in the void volume (V_0-material) The other component protein AA, was considerably retarded, corresponding to a

TABLE 1 *Source of Tissues and Yield of Water-extractable Materials*

Source of tissue	Diagnosis	Organ	Yield of material extracted with water mg	Yield of V_o-material§ %
J.L.	Ankylosing spondylitis/amyloidosis	Liver	1 100	40
J.L.	Ankylosing spondylitis/amyloidosis	Spleen	900	41
T.H.	Juvenile rheumatoid arthritis/amyloidosis	Liver	500	53
J.B.	Waldenström's macroglobulinaemia/amyloidosis	Liver	700	19
A.R.	Primary amyloidosis	Spleen	1,000	23
N.N.	Normal Control	Liver	310	95
N.N.	Normal Control	Spleen	250	92

* Starting material 20 grams of fresh tissue

§ Determined by estimation of the area of the protein peaks monitored at 280 nm. The absorptions at 280 nm were recorded on a logaritmic scale and areas of the peaks determined after conversion of the logaritmic recording to a linear one.

munoglobulin light chain nature (8) and is connected mainly with primary or myeloma associated amyloidosis. The other subunit, the amyloid protein AA (for nomenclature, see reference 2) is of unknown origin (3 4 6 15 16 17 24) and is found at high concentration particularly but not exclusively in secondary amyloidosis (3 16) and in experimental amyloidosis in various animals (7 14 23). In addition, a material of higher molecular weight, the void volume or "V_o-material" is also consistently eluted during gel filtration of solubilized amyloid fibrils (3 11 13 17). It was the aim of this study to compare the amyloid V_o-material and a protein component prepared from corresponding normal organs using the same procedure for extraction.

MATERIALS AND METHODS

Amyloid fibril preparations. Amyloid fibrils were prepared from the liver of a patient (T.H.) with juvenile rheumatoid arthritis, from the spleen and liver of a patient (J.L.) with amyloidosis secondary to ankylosing spondylitis, and from the liver of a patient (J.B.) with amyloidosis associated with Waldenstrom's macroglobulinemia (Table 1). The amyloid fibrils from these patients have previously been shown to be of the protein AA type (13, 16, 24). Amyloid fibrils were also extracted from the spleen of a patient with primary amyloidosis of the immunoglobulin type (protein AR, V λ VI) previously described (13 25) (Table 1).

The amyloid fibrils were isolated according to *Pras et al.* (21 22) using extractions with distilled water after repeated washings of homogenized tissue with physiological saline until the optical density at 280 nm in the supernatant was negligible (11). The first distilled water supernatant was discarded while supernatants no 2 3 4 5 and 6 containing the extracted amyloid fibrils were lyophilized and kept at –20 C until used. For the preparation of high molecular weight V_o-material lyophilized amyloid fibrils were dissolved (20 mg/ml) in 6M guanidine/0 1 M dithiothreitol (DTT) and gel filtered on a Sephadex G-100 column equilibrated with 5M guanidine/1N acetic acid (15). Protein excluded by this column and thus eluted in the void volume (the V_o material) was collected, dialysed against distilled water and lyophilized. Amyloid fibrils were also treated with 0.1 N NaOH (2 mg/ml) for 2 hours and thereafter dialyzed against distilled water. The resulting alkali-degraded amyloid fibrils (DAM*) were used for immunization of rabbits to produce anti-amyloid antiserum, and for immunologic studies (11).

Normal tissue preparations. Normal spleen and liver tissues were obtained from a 32 years old previously healthy female (N.N.) who died in a traffic

* DAM = degraded amyloid fibrils.

TABLE 2 Amino Acid Composition of Crude and Fractionated Materials Obtained from Amyloid-laden and Normal Human Tissues

	Crude amyloid fibrils			Amyloid V -material					Crude normal tissue extract		Normal tissue V_0-material	
	J.L. Liver	J.B. Liver	A.R. Spleen	J.L. Spleen	J.L. Liver	T.H. Liver	A.R. Spleen	J.B. Liver	Spleen	Liver	Spleen	Liver
Asp	10.7	10.9	9.3	8.5	9.2	9.1	9.7	9.5	7.7	9.4	8.9	9.4
Thr	3.2	3.4	4.7	4.7	4.5	5.2	6.1	5.7	5.2	5.2	4.9	5.2
Ser	7.0	8.7	10.4	8.0	6.9	7.1	8.7	8.9	6.2	6.8	7.1	7.9
Glu	11.2	11.2	10.8	12.4	12.4	12.4	12.7	12.6	11.1	11.8	13.4	12.8
Pro	4.5	4.5	7.3	6.4	5.3	5.7	6.1	4.9	5.0	4.9	4.6	5.0
Gly	9.6	9.8	8.9	14.8	10.6	8.1	10.5	11.0	9.2	8.1	10.3	8.7
Ala	11.0	10.5	6.6	8.2	7.4	7.4	7.7	7.9	9.8	8.2	7.4	7.1
½ Cys		1.1	1.4	2.0	2.5	1.8		2.0	1.1	1.4	1.7	1.7
Val	4.7	4.5	6.9	4.5	5.0	5.4	5.9	3.3	5.6	5.9	5.3	5.7
Met	2.4	2.3	1.5	1.6	2.1	2.1		1.6	1.6	1.8	2.1	2.1
Ile	4.0	3.8	3.5	3.3	3.8	9	3.6	3.6	4.0	4.5	3.7	4.1
Leu	8.5	6.4	6.7	7.4	7.9	8.7	7.5	8.2	8.5	9.4	8.7	9.1
Tyr	4.5	4.2	3.9	2.2	2.7	2.8	4.4	2.7	5.1	3.1	3.6	3.1
Phe	6.9	7.2	3.0	4.5	5.9	6.6	4.1	5.5	5.2	4.4	4.1	4.5
His	1.8	1.9	2.1	1.8	1.9	2.1	2.0	2.0	2.0	2.2	2.0	2.1
Lys	4.7	5.0	4.4	4.9	5.2	5.8	5.8	5.7	9.7	7.6	6.3	6.8
Arg	7.3	7.1	3.9	3.0	3.6	5.3	5.0	4.8	7.1	5.5	5.0	5.0

lost during hydrolysis.

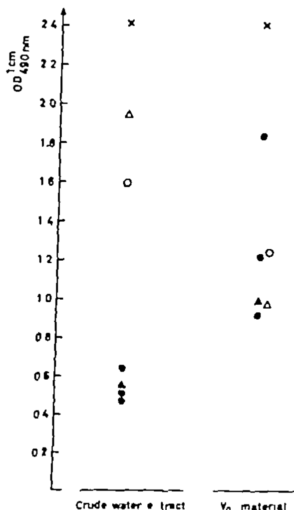


Fig 2 Congo-red absorption of crude water extracts from amyloidotic and normal tissues (left column) and the corresponding V_0-materials (right column) Amyloid or normal tissue materials were incubated with a 0 1 mg/ml solution of Congo-red in water and the absorption at 490 nm recorded *after removal of precipitated material by centrifugation.* Symbols ● liver amyloid, ▲ spleen amyloid, ○ normal liver Δ normal spleen, × *saline control. The most effective absorption was* achieved with crude (intact) amyloid fibrils.

much smaller molecular weight approximately 9 000 daltons. In contrast, only one major protein component, which corresponded to the void volume was obtained by gel filtration *of the normal tissue preparations* (Fig 1 b) No protein peak corresponding to amyloid proteins of low molecular weight (protein AA or protein subunits of the monoclonal immunoglobulin light chain type) could be detected in the *normal organs studied.* The yield of V_0-material obtained from amyloid fibrils and the normal tissue extracts is shown in Table 1 Fractions corresponding to the void volume were collected, dialysed extensively against distilled water and lyophilized

Congo-red binding Polarization microscopy revealed typical green birefringence when *tissue sections or lyophilized water extracts* from amyloid laden organs stained with Congo-red were examined, while no green birefringence could be seen if corresponding normal tissue sections or extracts were examined. Similar examination of the V_0 materials obtained by gel filtration did not show green birefringence, no matter whether the materials originated from amyloidotic or normal organs.

Suspensions of amyloid fibrils were highly effective in absorbing Congo-red (Fig 2) in *sharp contrast to the corresponding normal* tissue preparations where the absorbtion of Congo-red was minimal However no major differences in the absorbtion of Congo-red was observed when the V_0-materials from amyloid fibrils and normal extracts were compared (Fig 2) The sediments obtained *from the absorbtion experiments* were also

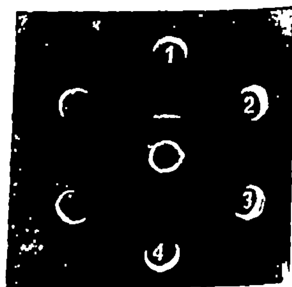


Fig 3 Double diffusion in agarose Anti-amyloid V_0-material in central well tested against crude DAM from T.H liver (*1*) crude water extract from normal liver treated with 0 1 N NaOH (2) amyloid V_0-material from R.A spleen (3) and V_0 *material from normal liver (4) The other peripheral* wells were filled with saline

conditions. This material, by us called the V_o-material (10, 13–15) has been less extensively studied. Some amino acid composition data and peptide map analyses have however been reported from different groups (3–15–17). In addition, previous immunologic studies (13–15) have shown evidence that the V_o-material is antigenically distinct from immunoglobulins and protein AA (13–15) as well as from hormone-like amyloid fibril sub-units (26).

The results reported here give evidence that a material which is antigenically and chemically similar if not identical, to the V_o-material of amyloid fibrils, can be isolated from normal tissues where no amyloid substance can be detected by sensitive histochemical techniques. The weak, or lacking, reactivity of the normal tissue preparations when tested against the anti-amyloid V_o-material may be explained by differences in the exposure of antigenic determinants involved in the reactions. It is thus conceivable, that the V_o-material found in amyloid fibril preparations, is a normal tissue constituent. The amino acid composition of our amyloid and normal tissue V_o-materials was also strikingly similar to a protein similarly extracted from pig liver and kidney reported by *Pras & Glynn* (19). The authors suggested that this protein is of connective tissue origin namely a reticulin protein.

The question remains to be answered whether the V_o-material of amyloid is an integral part of the fibril or it only represents a contaminating protein which is also easily suspended in water and is thus extracted together with the fibrils during the isolation procedures (10, 18). In this connection it should be noted that the amyloid V_o-material, as well as normal tissue preparations, absorbed Congo-red poorly as compared with the native amyloid fibrils, and that it did not show specific birefringence under crossed polars. Absorption of Congo-red is known to be a characteristic feature of amyloid fibrils (21). On the other hand, electron microscopic examination of amyloid fibrils isolated by the method described consistently showed very pure preparations of typical amyloid fibrils without significant amounts of non-fibrillar material, even in preparations where the V_o-material constituted up to 50 per cent of the total proteins (*Husby et al.* manuscript in preparation). Furthermore, very strong dissociating conditions are required in order to split the V_o-material from the amyloid subunit of smaller molecular weight indicating a close association between these two proteins. We conclude therefore that the V_o-material which seems to be a normal tissue constituent, most probably is an integral part of the amyloid fibrils. Another protein component of smaller molecular weight also seems to be required for the fibril formation. This latter protein may differ in different amyloid preparations. Until now it has been shown that immunoglobulin chains or fragments, protein AA, and endocrine hormone like polypeptides is found in amyloid fibrils together with the V_o-material. Studies are now undertaken where different combinations of isolated amyloid proteins in addition to related serum components (1–12, 14–16) are incubated under varying *in vitro* conditions to see whether fibrils ultrastructurally typical for the amyloid fibrils are formed.

The authors want to thank Miss *Brith Skrøgstad* for skilful technical assistance

REFERENCES

1. *Anders R. F. Natvig J. B., Michaelsen T. E. & Husby G.* Isolation and characterization of amyloid-related serum protein SAA as a low molecular weight protein. Scand. J. Immunol. 4 397–401 1975.
2. *Benditt E. P. Cohen A. S. Franklin, E. F., Glenner G. G., Husby G. Natvig, J. B. Ostermann, E. F. & Wegelius O.*: Nomenclature for amyloid proteins and related serum components. In *Wegelius O. & Pasternack, A.* (Eds.) Amyloidosis, Academic Press, New York, London, In press.
3. *Benditt E. P. & Eriksen N.*: Chemical similarity among amyloid substances associated with long standing inflammation. Lab. Invest. 26 615–625 1972.
4. *Benditt E. P. Eriksen N., Hermodson M. A.*

subjected to polarization microscopy Again typical green birefringence was seen when sediments containing crude amyloid fibrils were examined while neither of the sediments from crude normal tissue extracts, nor the V_0 materials from amyloidotic or normal tissues showed such birefringence

Immunologic studies Rabbits had been immunized weekly or biweekly for up to 8 months with "native" or NaOH treated water extracts from normal tissues. However a precipitating antiserum was not obtained in any of the animals. An anti-amyloid antiserum (Anti DAM I) which after absorption had previously been shown to have specificity for the V_0-material of most human amyloid preparations studied by us (13) was therefore used for the immunodiffusion studies. A precipitation line of identity with that of the crude DAM as well as the V_0-material from the amyloid preparation used for immunization was obtained by all the alkali degraded crude amyloid fibrils (DAM) when these were tested at a concentration of 2 mg/ml (Fig 3) A precipitation reaction of identity with these preparations was also obtained if the alkali treated water extracts from the normal organs were used (Fig 3) However a concentration of 8 mg/ml or more of the lyophilized normal tissue extracts was necessary to obtain a precipitation reaction with antibodies to anti amyloid V_0 material. No precipitation line was obtained by the normal tissue V_0 materials when tested against antiamyloid V_0 material at a concentration of up to 10 mg/ml of lyophilized material. No precipitation reaction was obtained by immunodiffusion between any normal tissue preparation and antisera to amyloid fibril subunit proteins AA and AR (VλV) or the amyloid P-component

Amino acid analyses The amino acid composition of the crude amyloid fibrils, amyloid V_0 material and crude water extracts and V_0 material from corresponding normal tissues are shown in Table 2 Comparison of crude amyloid fibrils and water extracts from normal tissues revealed differences in the content of aspartic acid leucine, phenylalanine and lysine. Crude amyloid and amyloid V_0-material were also different in the content of the amino acids alanine leucine tyrosine, phenylalanine and arginine. These differences were obviously due to the separation of the low molecular weight amyloid proteins from the V_0-material (Fig 1a) The amino acid composition of the V_0-material of amyloid was very similar to that of the crude normal tissue extracts as well as the V_0-material obtained from the normal tissues.

Partial N terminal amino acid sequence analyses were also performed. However a high degree of heterogeneity was observed in all the crude preparations as well as in the V_0 materials from amyloidotic and normal tissues except in amyloid V_0-materials from T.H liver and J L. spleen, in which the N-terminus was blocked A very low yield of amino acids at the N terminal (3–4 nanomol/mg dry weight of protein) strongly indicated, however that the majority of the protein of the V_0-material from both amyloidotic and normal tissues had a blocked N terminus, and that the apparent heterogeneities were due to minor amounts of contaminating polypeptides.

DISCUSSION

During the recent years much knowledge has been obtained concerning the nature of amyloid fibrils in different clinical cathegories of human amyloidosis as well as in various experimental models in animals (7 14 23) The investigations have so far been focused mainly on the different, homogeneous, low molecular weight proteins which have been shown to be major subunits of the fibrils, namely immunoglobulin light chains (8 13 24) protein AA (3 4 6 15 16 17 24) and recently a calcitonin like protein which has been characterized as a major component of amyloid found locally in medullary carcinomas of the thyroid (26) Furthermore a protein material of much higher molecular weight, is also consistently eluted when purified amyloid fibrils are solubilized and subjected to gel filtration under dissociating

conditions. This material, by us called the V_{σ}-material (10 13 15) has been less extensively studied. Some amino acid composition data and peptide map analyses have however been reported from different groups (3 15 17). In addition, previous immunologic studies (13 15) have shown evidence that the V_{σ}-material is antigenically distinct from immunoglobulins and protein AA (13 15) as well as from hormone like amyloid fibril sub-units (26).

The results reported here give evidence that a material which is antigenically and chemically similar if not identical, to the V_{σ}-material of amyloid fibrils, can be isolated from normal tissues where no amyloid substance can be detected by sensitive histochemical techniques. The weak, or lacking reactivity of the normal tissue preparations when tested against the anti-amyloid V_{σ}-material may be explained by differences in the exposure of antigenic determinants involved in the reactions. It is thus conceivable, that the V_{σ}-material found in amyloid fibril preparations, is a normal tissue constituent. The amino acid composition of our amyloid and normal tissue V_{σ}-materials was also strikingly similar to a protein similarly extracted from pig liver and kidney reported by *Pras & Glynn* (19). The authors suggested that this protein is of connective tissue origin, namely a reticulin protein.

The question remains to be answered whether the V_{σ}-material of amyloid is an integral part of the fibril or it only represents a contaminating protein which is also easily suspended in water and is thus extracted together with the fibrils during the isolation procedures (10 18). In this connection it should be noted that the amyloid V_{σ}-material, as well as normal tissue preparations, absorbed Congo-red poorly as compared with the "native" amyloid fibrils, and that it did not show specific birefringence under crossed polars. Absorption of Congo-red is known to be a characteristic feature of amyloid fibrils (21). On the other hand, electron microscopic examination of amyloid fibrils isolated by the method described, consistently showed very pure preparations of typical amyloid fibrils without significant amounts of non-fibrillar material, even in preparations where the V_{σ}-material constituted up to 50 per cent of the total proteins (*Husby et al.*, manuscript in preparation). Furthermore, very strong dissociating conditions are required in order to split the V_{σ}-material from the amyloid subunit of smaller molecular weight indicating a close association between these two proteins. We conclude therefore that the V_{σ}-material which seems to be a normal tissue constituent, most probably is an integral part of the amyloid fibrils. Another protein component of smaller molecular weight also seems to be required for the fibril formation. This latter protein may differ in different amyloid preparations. Until now it has been shown that immunoglobulin chains or fragments, protein AA, and endocrine hormone-like polypeptides is found in amyloid fibrils together with the V_{σ}-material. Studies are now undertaken where different combinations of isolated amyloid proteins in addition to related serum components (1 12, 14 16) are incubated under varying *in vitro* conditions to see whether fibrils ultrastructurally typical for the amyloid fibrils are formed.

The authors want to thank Miss *Brith Skrogstad* for skilful technical assistance.

REFERENCES

1 *Anders, R F., Natvig, J B., Michaelsen T E. & Husby G* Isolation and characterization of amyloid-related serum protein SAA as a low molecular weight protein. Scand, J Immunol. 4 397–401 1975.
2. *Benditt E. P Cohen A. S Franklin E. F Glenner G G., Husby G., Natvig J B., Ossermann E. F & Westermark O.* Nomenclature for amyloid proteins and related serum components. In *Wegelius O & Pasternack A* (Eds.) Amyloidosis, Academic Press, New York, London, In press.
3 *Benditt E. P & Eriksen, N.* Chemical similarity among amyloid substances associated with long standing inflammation. Lab. Invest. 26 615–625, 1972.
4 *Benditt E. P., Eriksen N Hermodson M A*

& *Ericsson L. H* The major proteins of human and monkey amyloid substances Common properties including unusual N terminal amino acid sequences. FEBS Letters *19* 169–173 1971

5 *Cathcart E. S Wollheim F A & Cohen A S* Plasma protein constituents of amyloid fibrils. J Immunol. *99* 376–385 1967

6 *Ein D Kimura S & Glenner G G* An amyloid fibril protein of unknown origin Partial amino acid sequence analysis. Biochem. Biophys. Res. Comm. *46* 498–500 1972.

7 *Eriksen N Ericsson L. H Pearsall N Lagunoff D & Benditt E P* Mouse amyloid protein AA Homology with nonimmunoglobulin protein of human and monkey amyloid substance. Proc Nat. Acad. Sci. *73* 964–967 1976

8 *Glenner G G., Terry W D & Isersky C* Amyloidosis Its nature and pathogenesis. Semin Hematol *10* 65–86 1973

9 *Harada M Isersky C Cuatrecasas P Page D Bladen H A., Eanes E D Keiser H R & Glenner G G* Human amyloid protein Chemical variability and homogeneity J Histochem. Cytochem. *19* 1–15 1971

10 *Husby G* Amyloidosis in rheumatoid arthritis Ann Clin. Res. *7* 154–167 1975

11 *Husby G & Natvig J B* Individual antigenic specificity and cross-reactions among amyloid preparations from different individuals. Clin. exp Immunol *10* 635–647 1972

12 *Husby G & Natvig J B* A serum component related to nonimmunoglobulin amyloid protein AS a possible precursor of the fibrils. *J Clin Invest. 53* 1054–1061 1974

13 *Husby G Natvig J B & Sletten K* New third class of amyloid fibril protein. J exp. Med *139* 773–778 1974

14 *Husby G Natvig, J B Sletten K Nordstoga, K & Anders R F* An experimental model in mink for studying the relation between amyloid fibril protein AA and the related serum protein SAA. Scand. J Immunol *4* 811–816 1975

15 *Husby G Sletten K Michaelsen T E & Natvig J B* Antigenic and chemical characterization of non-immunoglobulin amyloid proteins. Scand J Immunol *1* 393–400 1972.

16 *Husby G Sletten K Michaelsen T E & Natvig J B.* Amyloid fibril protein subunit, "protein AS" distribution in tissue and serum in different clinical types of amyloidosis including that associated with myelomatosis and Waldenstrom s macroglobulinaemia. Scand J Immunol. *2* 395–404 1973

17 *Levin M Franklin E C., Frangione B. & Pras M* The amino acid sequence of a major non-immunoglobulin component of some amyloid fibrils. J clin. Invest. *51* 2773–2776, 1972.

18 *Natvig, J B., Husby G Sletten K. & Michaelsen T* Structural and antigenic classification of amyloid fibril proteins in primary myeloma associated and secondary amyloidosis. In *Wegelius O & Pasternack A* (Eds.) Amyloidosis. Academic Press, New York, London, In press.

19 *Pras M & Glynn L. E.* Isolation of a noncollagenous reticulin component and its primary characterization. Br J Exp Pathol. *54* 449–456 1973

20 *Pras M & Reshef T.* The acid-soluble fraction of amyloid—a fibril forming protein Biochim. biophys. Acta (Amst.) *271* 193–203 1972.

21 *Pras M., Schubert M Zucker-Franklin, D Rimon A & Franklin E C.* The characterization of soluble amyloid prepared in water J clin. Invest. 47 924–933 1968.

22 *Pras M Zucker-Franklin D Rimon, A. & Franklin E C* Physical, chemical and ultrastructural studies of water-soluble human amyloid fibrils. J Exp. Med *130* 777–795 1969.

23 *Skinner M., Cathcart E S Cohen A. S & Benson M D* Isolation and identification by sequence analysis of experimentally induced guinea pig amyloid fibrils. J Exp Med. *140* 871–876 1974

24 *Sletten K & Husby G* The complete amino acid sequence of non-immunoglobulin amyloid fibril protein AS in juvenile rheumatoid arthritis Eur J Biochem. *41* 117–125 1974

25 *Sletten K Husby G & Natvig, J B.* N terminal amino acid sequence of amyloid fibril protein AR. Prototype of a new λ variable subgroup V λV Scand. J Immunol *3* 833–836 1974

26 *Sletten K Westermark P & Natvig, J B.* Characterization of amyloid fibril proteins from medullary carcinoma of the thyroid. J Exp. Med. *143* 993–998, 1976.

Acta path. microbiol. scand. Sect. C, 85: 161–168, 1977

PHAGOCYTOSIS OF HEAT-KILLED RADIOLABELLED MYCOBACTERIA IN HUMAN MONONUCLEAR PHAGOCYTES CULTURED *IN VITRO*

K. E. VIKEN, G. UNSGAARD and A. ØDEGAARD

Trondheim Regional Hospital, Department of Medicine, Section for Haematology and Immunology, N-7000 Trondheim, Norway

Viken, K. E., Unsgaard, G. & Ødegaard, A. Phagocytosis of heat-killed, radiolabelled mycobacteria in human mononuclear phagocytes cultured *in vitro*. Acta path. microbiol. scand. Sect. C, 85: 161–168, 1977.

Human mononuclear phagocytes cultured *in vitro* for 8 days were exposed to ^{125}I-labelled, heat-killed *Mycobacterium trivlale*. The microorganisms were apparently engulfed, but no digestion occurred within a period of 16 days after the engulfment, measured as release of radioactivity to the medium and observed microscopically. Attempts were made to stimulate intracellular digestion of the bacteria. Pre-incubation with BCG-stimulated lymphocytes or with supernatants from BCG-stimulated lymphocyte cultures did not increase the digestive ability of the cells. However, pre-incubation with BCG-stimulated lymphocytes or with supernatants caused detachment of the cells during the following digestion period, probably due to a cytotoxic effect of autologous, transformed lymphocytes on macrophages. When the macrophages were cultured in the presence of autologous lymphocytes and BCG, a similar effect was found.

Key words: Mycobacteria; mononuclear phagocytes; lymphocytes; *in vitro*.

K. E. Viken, Trondheim Regional Hospital, Department of Pathology, N-7000 Trondheim, Norway.

Received 30.ix.76. Accepted 25.xi.76.

It is anticipated that the course of a granulomatous inflammation such as tuberculosis is dependent on the ability of the mononuclear phagocyte to eliminate the infective agent. Although monocytes and macrophages are revealed as the effector cells in intracellular infections, immunologically comitted lymphocytes are important as macrophage activators. These cells produce mediators that bring phagocytes to an infective focus and stimulate them metabolically, thus creating the conditions needed to interfere with the survival of parasites (9). *Failure to kill* organisms is the major cause of prolonged inflammation, but inability to degrade dead organisms to soluble products may be a contributory factor (14).

In vivo and *in vitro* experiments have demonstrated that activated macrophages are able to increase their bactericidal activity towards intracellular parasites such as *Mycobacterium tuberculosis* (5, 11), *Listeria monocytogenes* (6, 8, 13) and *Mycobacterium le*

& *Ericsson L. H* The major proteins of human and monkey amyloid substances Common properties including unusual N-terminal amino acid sequences. FEBS Letters *19* 169–173 1971

5 *Cathcart E. S Wollheim F A & Cohen A S* Plasma protein constituents of amyloid fibrils. J Immunol. *99* 376–385 1967

6 *Ein D Kimura S & Glenner G G* An amyloid fibril protein of unknown origin Partial amino acid sequence analysis. Biochem. Biophys. Res. Comm. *46* 498–500 1972.

7 *Eriksen N Ericsson L. H Pearsall N., Lagunoff D & Benditt E. P* Mouse amyloid protein AA Homology with nonimmunoglobulin protein of human and monkey amyloid substance. Proc, Nat. Acad. Sci. *73* 964–967 1976

8. *Glenner G G Terry W D & Isersky C* Amyloidosis Its nature and pathogenesis. Semin. Hematol *10* 65–86 1973

9 *Harada M Isersky C Cuatrecasas P Page D., Bladen H A., Eanes E D., Keiser H R & Glenner G G* Human amyloid protein Chemical variability and homogeneity J Histochem. Cytochem. *19* 1–15 1971

10 *Husby G* Amyloidosis in rheumatoid arthritis. Ann. Clin. Res. *7* 154–167 1975

11 *Husby G & Natvig J B* Individual antigenic specificity and cross-reactions among amyloid preparations from different individuals. Clin. exp Immunol *10* 635–647 197?

12. *Husby G & Natvig J B* A serum component related to nonimmunoglobulin amyloid protein AS a possible precursor of the fibrils. J Clin. Invest. *53* 1054–1061 1974

13 *Husby G Natvig J B & Sletten K* New third class of amyloid fibril protein. J exp. Med *139* 773–778, 1974

14 *Husby G Natvig, J B. Sletten K Nordstoga K & Anders R F* An experimental model in mink for studying the relation between amyloid fibril protein AA and the related serum protein SAA. Scand. J Immunol *4* 811–816 1975

15 *Husby G Sletten K. Michaelsen T E. & Natvig J B* Antigenic and chemical characterization of non-immunoglobulin amyloid proteins. Scand. J Immunol. *1* 393–400 1972.

16 *Husby G Sletten K Michaelsen T E. & Natvig J B.* Amyloid fibril protein subunit, "protein AS" distribution in tissue and serum in different clinical types of amyloidosis including that associated with myelomatosis and Waldenstrom s macroglobulinaemia. Scand. J Immunol. *2* 395–404 1973

17 *Levin M Franklin E C Frangione B & Pras M* The amino acid sequence of a major non-immunoglobulin component of some amyloid fibrils. J clin. Invest. *51* 2773–2776 1972.

18. *Natvig J B Husby G., Sletten K. & Michaelsen T* Structural and antigenic classification of amyloid fibril proteins in primary myeloma associated and secondary amyloidosis. In *Wegelius O & Pasternack A.* (Eds) *Amyloidosis. Academic Press,* New York, London, In press.

19 *Pras M & Glynn L. E.* Isolation of a noncollagenous reticulin component and its primary characterization. Br J Exp. Pathol. *54* 449–456 1973.

20. *Pras M & Reshef T* The acid-soluble fraction of amyloid—a fibril forming protein Biochim. biophys. Acta (Amst.) *271* 193–203 1972

21 *Pras M., Schubert M Zucker-Franklin D Rimon A & Franklin E. C* The characterization of soluble amyloid prepared in water. J clin. Invest. 47 924–933 1968.

22. *Pras M., Zucker Franklin D Rimon A & Franklin E. C* Physical chemical and ultrastructural studies of water-soluble human amyloid fibrils. J Exp. Med *130* 777–793, 1969

23 *Skinner M., Cathcart E. S Cohen A S & Benson M D* Isolation and identification by sequence analysis of experimentally induced guinea pig amyloid fibrils. J Exp. Med. *140* 871–876 1974

24 *Sletten K. & Husby G* The complete amino acid sequence of non-immunoglobulin amyloid fibril protein AS in juvenile rheumatoid arthritis Eur J Biochem. *41* 117–125 1974

25 *Sletten K., Husby G & Natvig, J B.* N terminal amino acid sequence of amyloid fibril protein AR. Prototype of a new λ variable subgroup V λV Scand. *J Immunol 3* 833–836 1974

26 *Sletten K Westermark P & Natvig, J B* Characterization of amyloid fibril proteins from medullary carcinoma of the thyroid. J Exp. Med *143* 993–998, 1976

digestion capacity was registered after 4 days, measured as release of radioactivity to the medium.

Test procedure 2. Macrophages were cultured on coverslips for 8 days under normal culture conditions. The culture medium was removed and 2.5 ml culture medium (with AB-serum) containing freshly separated autologous mononuclear blood cells in concentration of 10^6 cells per ml and 10^7 BCG bacilli per ml were added to the dishes. Cell suspensions without BCG served as controls. On the 13th day of culture (5 days after the addition of lymphocytes + antigen) 0.5 μCi per ml of ³H-thymidine was added to some of the culture dishes. Eighteen hours later these cultures were harvested as described in test procedure 1. In the rest of the cultures the digestion capacity was registered after 4 and 12 days, as described earlier.

Test procedure 3. Cell free supernatants from BCG-stimulated and non-stimulated lymphocyte cultures, prepared as described in test procedure 1, were harvested after 2 and 6 days of culture. The supernatants were dialyzed against 40 per cent solution of Ficoll (Pharmacia, Sweden, mol. wt. 4000,000) in distilled water for 24 hours. The non-dialyzable fraction (concentrated about 100 times) was diluted 1:100 in normal culture medium to obtain culture medium containing lymphokines in about the same concentration as in the lymphocyte cultures, with fresh culture medium components. This culture medium was added to homologous macrophage cultures after the engulfment of radiolabelled mycobacteria, and was thus present during the digestion period from the 8th to the 20th day of culture. The release of ¹²⁵Iodine to the medium was registered as described earlier.

Statistics

The experiments were performed in triplicate culture dishes or in triplicate culture tubes. In each experiment, the mean values from replicates were calculated and the results were expressed as percentages of control values ± standard deviation (SD). The p-values were calculated using the Wilcoxon Two-Sample test.

RESULTS

Measurement of the labelling kinetics showed that a maximum of 5 per cent of the radioactivity added was fixed to the bacilli after a labelling period of 4 hours followed by washing 4 times in saline. After the washing procedure more than 95 per cent of the radioactivity was found in the sediment obtained by centrifugation at 2000 *G* for 20 minutes. The passive release of ¹²⁵Iodine to the culture medium during incubation was approximately 30 per cent after 4 to 16 days (Fig. 1).

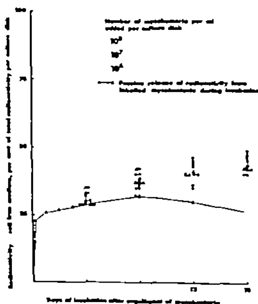


Fig. 1. The digestion capacity of macrophages, measured as release of radioactivity to the medium at different intervals after addition of radiolabelled mycobacteria. The values plotted are the means of the results from each of 13 experiments performed in triplicate culture dishes.

The amount of attached/engulfed radiolabelled mycobacteria showed a linear correlation (correlation coefficient 0.99) to the number of bacilli added in concentrations of 10^6 to 10^8 bacilli per ml (Fig. 2).

Microscopy of Ziehl-Neelsen stained cells revealed intact microorganisms after 16 days of incubation. The mycobacteria were situated around the cell nuclei, leaving the outer rim of cytoplasm free from bacilli (Fig. 3). Following the addition of bacilli the macrophages had become round and showed degranulation of the cytoplasm, as observed by phase contrast microscopy.

No definite intracellular digestion of heat killed mycobacteria could be registered during an incubation period of 8 days, measured as release of ¹²⁵Iodine to the medium (Fig. 1). After 12 and 16 days, the release of radioactivity increased slightly as compared to the

praemunium (7) In a guinea pig system, functions such as macrophage adherence phagocytosis, spreading and motility were all found to be enhanced after the addition of supernatants from stimulated lymphocyte cultures (10)

Non specific activated human macrophages obtained by culturing for 8 days in the presence of serum showed improved ability to digest heat killed radiolabelled *Candida albicans* (16 18) Most of the experimental work concerning the intracellular fate of mycobacteria has been done on animals The aim of this work was to study the intracellular degradation of killed mycobacteria in non specific activated *human* macrophages cultured *in vitro* using a quantitative procedure for registration of phagocytosis. Some experiments were designed to look for specific induction of intracellular degradation of killed mycobacteria in human macrophages by BCG stimulated lymphocytes

MATERIALS AND METHODS

Human mononuclear blood cells were separated from venous blood from different healthy donors who showed a positive tuberculin reaction after BCG-vaccination (3) The cells were cultured either as monolayers on glass coverslips in petri dishes (referred to as macophage cultures) or in Kimax tubes (referred to as lymphocyte cultures) The macrophages on coverslips were cultured in medium R.P.M.I 1640 supplemented by 25 per cent human pooled A-serum I glutamin and gentamicin (complete culture medium) Details of the culturing of monocytes/macrophages and lymphocytes have been reported previously (16 17 18) The cells in the macrophage cultures showed a marked ability to engulf and digest *Candida albicans* (16 18)

Mycobacteria

Mycobacterium triviale (Runyon group III non-photochromogen) was heat killed at 60 C for 2 hours and radiolabelled with [125]Iodine by means of electrolysis, as described for labelling of heat killed *Candida albicans* (15) The number of bacilli was counted in Bürker-chambers.

Phagocytosis of Mycobacteria

Mononuclear blood cells were cultured on coverslips for 8 days *in vitro* (referred to as macrophages) The culture medium was removed and different concentrations of labelled mycobacteria suspended in complete culture medium were added to the dishes. The cells were incubated further for 1 hour In the engulfment experiments, the coverslips were then rinsed 12 times in Hanks Balanced Salt Solution (BSS) and the amount of radioactivity on the coverslips was counted in a Wallac G.M gamma scintillation counter In the digestion experiments the non-attached/engulfed bacilli were removed by washing in complete medium The coverslips were then transferred to new petri dishes and fresh culture medium was added. The cultures were harvested after 4 8, 12 and 16 days of incubation and the radioactivity on the coverslips, in the sediment and in the cell free medium, obtained after centrifugation at 2000 G for 20 min, was measured separately (16) The digestion capacity was defined as the percentage of radioactivity found in the cell fre medium.

Cell Morphology

Some coverslips were removed at different intervals after the addition of mycobacteria in the digestion experiments The cell layer was washed in Hanks BSS fixed in ethanol and stained by Ziehl-Neelsen's method and microphotographs were made Intact bacilli were recognised as non-fragmented homogenously stained acid-resistant bacilli Furthermore the morphology of living cells at different stages of phagocytosis was observed in microchambers, using a phase contrast microscope (Leitz, W G) (18)

Attempt to Stimulate Intracellular Digestion of Mycobacteria

Test procedure 1 Mononuclear blood cells were cultured in Kimax tubes in volumes of 2.5 ml culture medium containing 10^6 cells per ml BCG-bacilli (Statens Seruminstitut Denmark) in a concentration of 10^7 bacilli per ml were added per culture tube On the 5th day of culture 0.5 µCi per ml of ^{3}H thymidine was added to some of the culture tubes. Eighteen hours later these tubes were harvested using a Titertek multiple cell harvester (Flow Scotland) and the radioactivity incorporated in the cells was measured in a liquid scintillation counter (Isocap/300 Nuclear Chicago, U.S.A.) The rest of the cultures were centrifuged at 400 *G* for 10 minutes. The supernatants were added to autologous macrophages cultured for 6 days *in vitro* on coverslips The cells in the culture tubes were resuspended in fresh culture medium and this suspension was added to other 6-day old autologous macrophage cultures. After a further culture period of 2 days, the culture media were removed and the cells were exposed to 10 radio-labelled mycobacteria per ml for 1 hour The

TABLE 2. *The Digestion of Mycobacteria in Human Macrophages after Exposure to Autologous Lymphocytes and BCG*

Test media/ Incubation time	Radioactivity on coverslips, per cent	Radioactivity in sediment, per cent	Radioactivity in cell-free medium, per cent	Total radioactivity per culture dish, per cent of controls
Autologous lymphocytes with BCG/4 days	17 ± 5	39 ± 7	44 ± 2	58 ± 12
Autologous lymphocytes without BCG/4 days	64 ± 3	3 ± 1	33 ± 3	100
Autologous lymphocytes with BCG/12 days	3 ± 1	55 ± 3	53 ± 3	48 ± 2
Autologous lymphocytes without BCG/12 days	48 ± 4	9 ± 4	43 ± 1	100

The figures listed are the means ± SD of the results of one experiment carried out in 6-plicate culture dishes, as described in test procedure 2. The radioactivities in the culture compartments are expressed as percentages of the total radioactivity per culture dish. Two other experiments showed similar results.
³H-thymidine-incorporation in lymphocytes. Cultured with BCG: 11 ± 6, cultured without BCG: 9 ± 7 ct/min × 10 (means ± SD of the results from 3 experiments carried out in triplicate culture dishes as described in test procedure 2)

TABLE 3. *The Digestion of Mycobacteria in Human Macrophages in the Presence of Supernatants from BCG-stimulated Lymphocyte Cultures*

Test media/ Incubation time	Radioactivity on coverslips, per cent	Radioactivity in sediment, per cent	Radioactivity in cell-free medium, per cent
Supernatants from BCG-stimulated lymphocyte cultures/4 days	68 ± 6	8 ± 5	24 ± 2
Supernatants from non-stimulated lymphocyte cultures/4 days	70 ± 6	7 ± 5	23 ± 1
Supernatants from BCG-stimulated lymphocyte cultures/12 days	46 ± 13	24 ± 11	31 ± 3
Supernatants from non-stimulated lymphocyte cultures/12 days	55 ± 7	16 ± 6	30 ± 2

The figures listed are the means ± SD of the results of 11 experiments carried out in triplicate culture dishes, as described in test procedure 3. The values are presented as percentages of total radioactivity per culture dish. Macrophages from 11 individuals were used.

(Table 1). The total radioactivity per culture dish was slightly reduced and the radioactivity in the sediment increased from 4 to 33 per cent ($p<0.01$) when macrophages had been pre-incubated with BCG-stimulated lymphocytes, thus indicating increased detachment of the macrophages both during the pre-incubation period and during the digestion period. After pre-incubation with supernatants from BCG-stimulated lymphocyte cultures, the radioactivity per culture dish increased as compared to controls ($p<0.01$) (Table 1). The radioactivity in the sediment increased during the digestion period from 3 to 10 per cent ($p<0.01$) (Table 1). The lymphocytes responded to BCG-

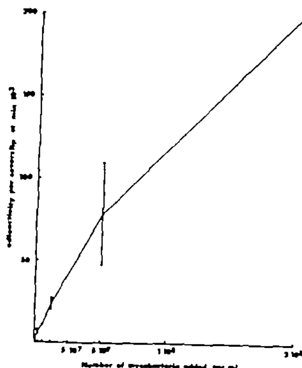


Fig 2 Radioactivity on coverslips containing 8-day-old macrophages after 1 hour's incubation with different concentrations of radiolabelled mycobacteria. The results are expressed as means ± SD of the data from 4 experiments and from one single experiment, carried out in triplicate culture dishes.



Fig 3 Macrophage exposed to 10^7 mycobacteria per ml on the 8th day of culture followed by further incubation for 16 days before fixation and staining with Ziehl-Neelsen's stain × 730.

passive release. Variations in the number of bacilli added from 10^6 to 10^8 bacilli per ml did not influence the results.

Pre-incubation with BCG-stimulated lymphocytes and supernatants from such cultures for 2 days before the addition of mycobacteria slightly increased the release of ^{125}Iodine to the cell free medium from 27 to 35 per cent and from 28 to 31 per cent, respectively

TABLE 1 *The Digestion of Mycobacteria in Human Macrophages after Pre-incubation with BCG-stimulated and Non stimulated Lymphocytes and Supernatants from BCG-stimulated and Non-stimulated Lymphocyte Cultures*

Test Media	Radioactivity on coverslips, per cent	Radioactivity in sediment, per cent	Radioactivity in cell-free medium, per cent	Total radioactivity per culture dish, per cent of controls
BCG-stimulated lymphocytes*	32 ± 10	33 ± 11	35 ± 6	96 ± 16
Non-stimulated lymphocytes*	65 ± 5	4 ± 1	27 ± 5	100
Supernatants from BCG-stimulated lymphocyte cultures	59 ± 7	10 ± 3	31 ± 5	110 ± 8
Supernatants from non-stimulated lymphocyte cultures	69 ± 7	3 ± 2	28 ± 5	100

The figures listed are the means ± SD of the results from 5 experiments carried out in triplicate culture dishes, as described in test procedure 1 The radioactivity in the culture compartments is expressed as percentage of the total radioactivity per culture dish. Macrophages from 5 different individual were used

* ^{3}H-thymidine-incorporation BCG-stimulated lymhpocytes 190 ± 76 non-stimulated lymphocytes 3 ± 1 ct/min × 10^3 (means ± SD of the results from 3 experiments carried out in triplicate culture tubes as described in test procedure 1)

TABLE 2. *The Digestion of Mycobacteria in Human Macrophages after Exposure to Autologous Lymphocytes and BCG*

Test media/ Incubation time	Radioactivity on coverslips, per cent	Radioactivity in sediment, per cent	Radioactivity in cell-free medium, per cent	Total radioactivity per culture dish, per cent of controls
Autologous lymphocytes with BCG/4 days	17 ± 5	39 ± 7	44 ± 2	58 ± 12
Autologous lymphocytes without BCG/4 days	64 ± 3	3 ± 1	33 ± 3	100
Autologous lymphocytes with BCG/12 days	5 ± 1	55 ± 3	53 ± 3	48 ± 2
Autologous lymphocytes without BCG/12 days	48 ± 4	9 ± 4	43 ± 1	100

The figures listed are the means ± SD of the results of one experiment carried out in 6-plicate culture dishes, as described in test procedure 2. The radioactivities in the culture compartments are expressed as percentages of the total radioactivity per culture dish. Two other experiments showed similar results.
^{3}H-thymidine-incorporation in lymphocytes. Cultured with BCG 11 ± 6 cultured without BCG 9 ± 7 ct/min × 10^3 (means ± SD of the results from 5 experiments carried out in triplicate culture dishes as described in test procedure 2)

TABLE 3. *The Digestion of Mycobacteria in Human Macrophages in the Presence of Supernatants from BCG-stimulated Lymphocyte Cultures*

Test media/ Incubation time	Radioactivity on coverslips, per cent	Radioactivity in sediment, per cent	Radioactivity in cell-free medium, per cent
Supernatants from BCG-stimulated lymphocyte cultures/4 days	68 ± 6	8 ± 5	24 ± 2
Supernatants from non-stimulated lymphocyte cultures/4 days	70 ± 6	7 ± 5	23 ± 1
Supernatants from BCG-stimulated lymphocyte cultures/12 days	46 ± 13	24 ± 11	31 ± 3
Supernatants from non-stimulated lymphocyte cultures/12 days	55 ± 7	16 ± 6	30 ± 2

The figures listed are the means ± SD of the results of 11 experiments carried out in triplicate culture dishes, as described in test procedure 3. The values are presented as percentages of total radioactivity per culture dish. Macrophages from 11 individuals were used.

(Table 1). The total radioactivity per culture dish was slightly reduced and the radioactivity in the sediment increased from 4 to 33 per cent ($p<0.01$) when macrophages had been pre-incubated with BCG-stimulated lymphocytes, thus indicating increased detachment of the macrophages both during the pre-incubation period and during the digestion period. After pre-incubation with supernatants from BCG-stimulated lymphocyte cultures, the radioactivity per culture dish increased as compared to controls ($p<0.01$) (Table 1). The radioactivity in the sediment increased during the digestion period from 3 to 10 per cent ($p<0.01$) (Table 1). The lymphocytes responded to BCG-

stimulation by cell proliferation measured as ^{3}H thymidine incorporation (Table 1)

Preincubation with autologous lymphocytes and BCG for 6 days (from the 8th to the 14th day of culture) increased the release of ^{125}Iodine to the medium from 33 to 44 per cent after 4 days and from 43 to 53 after 12 days of the following digestion period ($p<0.01$) The total radioactivity per culture dish was significantly decreased as compared to the activity of the controls thus indicating a decrease in the number of adhesive cells able to engulf mycobacteria (Table 2) This decrease in cell number was verified microscopically The radioactivity in the sediment increased from 3 to 39 per cent after 4 days and from 9 to 55 per cent after 12 days (Table 2) The lymphocytes harvested prior to the registration of phagocytosis did not proliferate after BCG-stimulation measured as ^{3}H thymidine-incorporation (Table 2)

The presence of dialyzed supernatants from BCG stimulated homologous lymphocyte cultures during the digestion period from the 8th to the 20th day of culture did not increase the digestion of mycobacteria in the mononuclear phagocytes. A slight increase in detachment of macrophages from 16 to 24 per cent ($p<0.01$) during the digestion period was observed (Table 3)

DISCUSSION

Human monocytes cultured *in vitro* differentiate into macrophages with increased ability for phagocytosis of *heat killed Candida* particles (16 18) This non specific "activation of the cells has been shown by *Cohn et al* (4) to involve synthesis of new lysosomes and is linked to the digestion of interiorized substrates.

In contrast to previous results, where an almost complete degradation of Candida particles was found (16) human macrophages cultured *in vitro* appeared to be unable to break down ingested heat killed mycobacteria measured as release of radioactivity However it is difficult, by the techniques used in this study to prove whether the bacteria are attached to the cell surface or interiorized The morphological changes observed in phase contrast microscopy and by examination of Ziehl Neelsen stained preparations indicate that the bacteria are engulfed by the macrophages. Ingestion of living and killed mycobacteria have been demonstrated by electron microscopy in studies with mouse peritoneal macrophages cultured *in vitro* (1)

In comparison with the passive release of radioactivity to the culture medium, a moderate increase in radioactivity was found in the medium after 12 and 16 days incubation of the macrophages following the addition of mycobacteria This increase may indicate digestion of engulfed bacteria. However this conclusion does not seem to be justified, because of the high degree of passive release from the mycobacteria

Spector et al (14) in a similar study using mouse peritoneal macrophages, observed a degradation of up to 50 per cent of ingested heat killed mycobacteria measured as release of ingested radioactivity to the medium. The discrepancy between their findings and those in the present study may be due to different properties of mouse peritoneal and human blood derived macrophages.

Tubercle bacilli proliferate in mononuclear phagocytes cultured *in vitro* *Patterson* & *Youmans* (11) have reported that lymphoid cells from tuberculin sensitive animals can suppress the growth of tubercle bacilli in cultures of infected macrophages. Accordingly immunologically committed lymphocytes are necessary to induce increased microbicidal activity in the macrophages.

In the present study attempts were made to stimulate the intracellular digestion of *killed* mycobacteria Neither pre-incubation with antigen stimulated lymphocytes nor pre incubation with or the presence of supernatants from such cultures increased the digestion The slight increase in release of radioactivity to the medium observed in some of the experiments may be due to increased release of radioactivity from lysed, detached cells.

Armstrong & Hart (1 2) observed that phagosomes containing viable *Mycobacterium tuberculosis* did not fuse with lysosomes unless they were pre-treated with specific antisera. Phagosomes containing non-viable bacilli did fuse with lysosomes. However degradation of the mycobacteria was not observed (1) This is in accordance with our findings. The lack of intracellular degradation of mycobacteria observed thus indicates that the cells do not synthetize lysosomal enzymes necessary for degradation. This resistance of mycobacteria to digestion may be due to the structure and composition of the bacilli, e.g. the large content of lipids. The apparent failure to induce production of lysosomal enzymes able to destroy these components may be due however to short stimulation and digestion periods or to failure to obtain *in vitro* conditions suitable for this complex cell interaction to take place.

Pre-incubation with BCG-stimulated lymphocytes caused detachment of the macrophages. This may be due to a cytotoxic effect of transformed lymphocytes on the cells in accordance with the findings of *Perlmann et al* (12) Pre-incubation with supernatants from BCG-stimulated lymphocytes slightly increased the number of engulfed particles. However these activated macrophages showed a greater tendency to detach during the following digestion period. The presence of supernatants from BCG-stimulated lymphocytes during the digestion period of 12 days was also found to increase the detachment of macrophages

The stimulating effect of BCG was registered as incorporation of H thymidine in proliferating lymphocytes. The presence of cultured autologous macrophages along with lymphocytes stimulated with BCG inhibited lymphocyte proliferation. This is in accordance with the results of other experiments using PHA, PPD allogenic lymphocytes and BCG to induce proliferation of lymphocytes in the presence of non-specific activated autologous macrophages (*Unsgaard* unpubl.) However the detachment of macrophages in such cultures was similar to that observed after preincubation with transformed lymphocytes. This finding may indicate that the cytotoxic effect of stimulated lymphocytes, and possibly also the macrophage activating effect, is not coupled to the blastoid transformation of the cells.

The technical assistance of Mrs. *B. H Hansen* Mrs. *A Remen* and Mrs. *B. Lippe* is gratefully acknowledged We are also indebted to Prof. *J O Lamvik* for assistance during the experimental work and the preparation of the manuscript. The work was supported by grants from the *Norwegian Research Council for Science and the Humanities* *the Norwegian Cancer Society* and the *Norwegian Society for Fighting Cancer*

REFERENCES

1 *Armstrong, J A. & Hart P D A cy* Response of cultured macrophages to *Mycobacterium tuberculosis* with observations on fusion of lysosomes with phagosomes. J Exp. Med. *134* 713–740, 1971

2. *Armstrong, J A. & Hart P D'Arcy* Phagosome-lysosome interactions in cultured macrophages infected with virulent tubercle bacilli. J Exp. Med. *142* 1–16 1975

3 *Boyum A* Separation of leucocytes from blood and bone marrow Scand. J Clin. Lab. Invest. *21* Suppl. 97 1968.

4 *Cohn Z. A & Benson B* The differentiation of mononuclear phagocytes. J Exp. Med. *121* 153–169 1965.

5 *Dannenberg, J A. M Ando M & Shima, K* Macrophage accumulation, division, maturation, and digestion and microbicidal capacities in tuberculous lesions. III The turnover of macrophages and its relation to their activation and antimicrobial immunity in primary BCG lesions and those of reinfection. J Immunol. *109* 1109–1121 1972.

6. *Fowles R. E., Fajardo I M Leibowitch, J L. & David J R.* The enhancement of macrophage bacteriostasis by products of activated lymphocytes. J Exp. Med. *138* 952 964 1973.

7 *Godal T Rees R. J W & Lamvik J O.,* Lymphocyte-mediated modification of blood derived macrophage function *in vitro*; inhibition of growth of intracellular mycobacteria with lymphokines. Clin. Exp. Immunol. *8* 625–637 1971

8 *Mackaness G B.,* Cellular resistance to infection. J Exp. Med. *116* 381–406 1962.

9 *Mackaness, G B.* Resistance to intracellular infection. J Infect. Dis. *123* 439–445 1971

10 *Nathan C F Karnovsky M L & David J R* Alterations of macrophage functions by mediators from lymphocytes. J Exp Med. *133* 1356–1376 1971

11 *Patterson R J & Youmans G P* Demonstration in tissue culture of lymphocyte mediated immunity to tuberculosis. Infect. Immun. *1* 600–603 1970

12. *Perlmann P Perlmann H & Holm G* Cytotoxic action of stimulated lymphocytes on allogenic and autologous erythrocytes. Science *160* 306–309 1968

13 *Simon H B & Sheagren J N* Cellular immunity *in vitro* I Immunologically mediated enhancement of macrophage bactericidal capacity J Exp Med *133* 1377–1389 1971

14 *Spector W G., Reichold N & Ryan G B* Degradation of granuloma-inducing microorganisms by macrophages. J Path. *101* 339–354 1970

15 *Viken K E* [125]I-labelling of *Candida albicans* by electrolysis. Acta path. microbiol. scand. Sect. B *82* 219–222, 1974

16 *Viken K E. & Odegaard A.*, Phagocytosis of heat-killed radiolabelled *Candida albicans* by human blood monocytes cultured *in vitro*. Acta path. microbiol. scand. Sect. B, *82* 235–244 1974

17 *Viken K E.* Effect of sodium-salicylate on the function of cultured, human mononuclear cells Acta path. microbiol. scand. Sect. C, *84* 465–470 1976

18. *Ødegaard A Viken K E & Lamvik J* Structural and functional properties of blood monocytes cultured *in vitro* Acta path. microbiol. scand. Sect. *82* 223–234 1974

Acta path. microbiol. scand. Sect. C, 85 169–177 1977

INHIBITORY EFFECTS OF PLASMA FROM URAEMIC PATIENTS ON HUMAN MONONUCLEAR PHAGOCYTES CULTURED *IN VITRO*

S. JØRSTAD and K. E. VIKEN

Trondheim Regional Hospital, Department of Medicine,
Sections for Haematology and Nephrology University of Trondheim, Trondheim, Norway

Jørstad, S. & Viken, K. E. Inhibitory effects of plasma from uraemic patients on human mononuclear phagocytes cultured *in vitro*. Acta path. microbiol. scand. Sect. C, 85: 169–177, 1977.

Human mononuclear phagocytes were cultured in plasma from uraemic patients. The presence of uraemic plasma during the engulfment or digestion of ^{125}I-labelled *Candida albicans* did not inhibit these functions in mononuclear phagocytes cultured for 8 days under normal conditions. When normal human macrophages were cultured in the presence of uraemic plasma for 2–4 days, a marked detachment of the cells from the glass coverslips was registered. The phagocytic function of the remaining cells was impaired. Creatinine, urea and methylguanidine in concentrations higher than those usually measured in plasma from uraemic patients did not influence the functional properties of the cells. The inhibitory effect of uraemic plasma on the mononuclear phagocytes is suggested as an explanation for the increased frequency of infections in uraemic patients.

Key words: Uraemia; mononuclear phagocytes; in vitro.

S. Jørstad, Department of Medicine, Section for Haematology and for Nephrology, Trondheim Regional Hospital, University of Trondheim, 7000 Trondheim, Norway.

Received 20.ix.76 Accepted 7.xii.76

Bacterial infections such as pneumonia and sepsis are a common problem in the management of patients suffering from serious renal failure. The phagocytic ability of granulocytes, blood monocytes and tissue macrophages are of importance for the outcome of bacterial infections (3).

Quadracci et al. (10) found that serum from patients with renal failure inhibited the growth of cultured fibroblasts. It has been demonstrated that the dialysate from uraemic patients suffering from neuropathia inhibited the growth of WD 38 cells (8). Further investigations have shown that a factor in uraemic plasma inhibited PHA-stimulated lymphocyte transformation (4, 7). *Hanicki et al.* (5) have shown recently that "middle molecules" of a molecular weight of 300–5000 from uraemic patients exert an inhibitory effect on PHA-stimulated lymphocytes, and that molecules in this range reduced the incorporation of amino-acids in unstimulated cells. *Burleson et al.* (1) found normal uptake of *Staphylococcus aureus* in leukocytes from uraemic patients cultured in non-uraemic

plasma, but impaired phagocytic capacity in the presence of autologous predialysis plasma. Using monocytes from uraemic patients as macrophage precursors *Urbanit & Sieberth* (15) found a decreased engulfment of IgG coated red cells in the presence of normal serum.

Publications on the behaviour of human cells under uraemic conditions are few and the causes of the increased frequency of infections in uraemic patients are generally unknown. The aim of the present work was, therefore, to investigate functional alterations in human mononuclear phagocytes caused by plasma from uraemic patients, using standardized test procedures.

MATERIALS AND METHODS

Venous blood was obtained from 18 uraemic patients with serum creatinine higher than 10 mg/100 ml.

Some patients were treated with haemodialysis, others were on dietary treatment. Only predialysis plasma was used from the dialysis patients. 0.05 mg heparin (Nyco A/S Norway) per ml was added to the samples. The blood cells were spun down and if not used immediately the plasma was stored in deep frozen state. As control pooled A-serum from healthy adults was used also with 0.05 mg heparin per ml added.

Cell Culture

The general methods for separation and culturing of human monocytes and for testing of phagocytosis have been reported in detail previously (16 18). Defibrinated venous blood from healthy adults was separated by gradient centrifugation using Lymphoprep (Nyco A/S Norway) as described by *Bøyum* (2). After washing in Hanks balanced salt solution (BSS) the mononuclear cells were suspended in three parts of the medium RPMI 1640 (Flow Scotland) supplemented with one part of pooled human A-serum, 0.1 mg l-glutamin per ml and 40 µg gentamicin per ml (complete medium). The solution was dispensed in volumes of 0.5 ml with 3×10^6 cells per ml on to glass coverslips (Leighton type 11×35 mm) in petri dishes (Nuclon, Denmark). The cells were cultured in a National CO incubator (USA) at 37 C with 5 per cent CO_2 in air and with 100 per cent humidity. After an incubation period of 90 min, the medium containing non-adherent cells (lymphocytes) was removed and 2.5 ml of fresh complete medium was added to each culture dish.

The medium was changed on the first and the fourth day of culture.

Candida albicans

Candida albicans (Department of Microbiology, Regional Hospital University of Trondheim) were heat killed at 60 C for 2 hours, and labelled with ^{125}I by means of electrolysis (17).

Test Procedure A

After washing the coverslips five times in Hanks BSS the cells were incubated with either 2.5 ml 100 per cent uraemic plasma, 2.5 ml uraemic plasma diluted to 25 per cent with RPMI 1640, 2.5 ml 100 per cent normal serum, or 2.5 ml 25 per cent normal serum diluted with RPMI 1640 from (a) 4th to the 8th day (b) 8th to the 10th day and c) 8th to the 12th day after start of culture.

Uraemic plasma with 25 per cent normal serum was also used as culture medium from the 4th to the 8th day with normal serum as control.

At the end of the incubation period the media were removed and each petri dish was incubated for 15 min with 2.5 ml complete medium containing 2×10^6 I labelled Candida particles per ml. The coverslips were then washed six times in complete medium and placed in new petri dishes, and fresh medium with 25 per cent normal pooled A-serum was added.

Mononuclear phagocytes were also exposed to equal amounts of uraemic serum/normal serum and normal plasma/normal serum from the 4th to the 8th day of culture as control experiments.

In some experiments, the number of cells on the coverslips was estimated microscopically using an inverted phase contrast microscope (Reichert, Austria) before the Candida particles were added.

The cultures were harvested after 24 hours. The medium was collected and centrifuged at 2000 G for 10 min. The radioactivity on each coverslip, in the supernatant and in the sediment, was counted using a Wallac GM gamma radiation counter (Finland).

Test Procedure B

Mononuclear phagocytes were cultured in complete medium for eight days. The media were then removed and the cells were incubated with either 100 per cent uraemic plasma, 100 per cent normal serum, 25 per cent uraemic plasma or normal serum diluted in RPMI 1640. The media contained 2×10^6 I-labelled Candida particles per ml. After incubation for 15 min the coverslips were washed 12 times in Hanks BSS and the radioactivity was registered.

TABLE 1 *Phagocytosis of Radiolabelled* Candida albicans *in 8-Day-Old Human Mononuclear Phagocytes Cultured in two Different Concentrations of Uraemic Plasma and Normal Serum from the 4th to the 8th Day before Start of Test*

Test media	Total radioactivity per culture dish	Distribution of radioactivity in different culture compartments after the digestion period from the 8th to the 9th day		
	Per cent *of controls*	Coverslips *per cent*	Sediment *per cent*	Cell-free medium *per cent*
Uraemic plasma 100 per cent	20.6 ± 8.8	43.2 ± 6.4	14.3 ± 3.4	42.5 ± 7.3
Normal serum 100 per cent	100*	26.4 ± 3.2	5.6 ± 1.2	68.0 ± 4.0
Uraemic plasma 25 per cent	55.9 ± 6.0	29.3 ± 4.1	9.6 ± 1.8	60.8 ± 5.4
Normal serum 25 per cent	100‡	26.3 ± 3.4	5.8 ± 1.1	67.9 ± 4.1

* Range 53–264 × 10³ ct/min radioactivity per culture dish.
‡ Range 87–291 × 10³ ct/min radioactivity per culture dish.
The values given are the means ± S.E.M. of the results from 10 experiments.
Each experiment was performed in triplicate culture dishes.
The radioactivity in the culture compartments is expressed as percentage of the total radioactivity in each culture dish

Test Procedure C

8-day-old macrophages were incubated with 2×10^6 radiolabelled Candida particles in fresh complete medium for 15 min. The coverslips were then washed 12 times in complete medium and placed in new petri dishes, and uraemic plasma and normal serum were added at concentrations as described above. The cultures were harvested after 24 hours, and the amount of radioactivity on the coverslips, in the supernatant and in the medium was registered.

Test Procedure D

Normal serum containing concentrations up to 990 mg/100 ml *of urea (Merck No 8487)* 33 mg/100 of creatinine (Merck No 5208) and 24 mg/100 ml of methylguanidine HCl (Sigma Chemical Company) was used as culture medium from the 4th to the 8th day after start of culture. The cells were then tested for phagocytic ability as described in test procedure A.

Test Procedure E

Mononuclear cells in a concentration of 5×10^6 per ml were suspended in Hanks BSS supplemented with Tris-HCl. To the cell suspension was added 10 µCi ^{51}Cr (Kjeller Norway) per ml and incubated for 30 min at 37 C (14). After radiolabelling the cells were washed twice in Hanks' BSS dispersed with 15×10^6 cells in uraemic plasma or normal serum in volumes of 2.5 ml per test tube. The amount of radioactivity was registered in the supernatant and in the sediment after incubation periods of 24 and 72 hours.

12*

Statistics

Each experiment was performed in triplicate culture dishes or in duplicate to quadriplicate culture tube. The results are given as the mean ± standard error of the mean (S.E.M.) unless otherwise stated. P-values were calculated using the Wilcoxon Two-sample test.

RESULTS

Test Procedure A

a) Table 1 shows the effect on macrophages cultured in uraemic plasma from the 4th to the 8th day of the culture period. A decrease in radioactive counts per culture dish to 20.6 and 55.9 per cent in 100 and 25 per cent uraemic plasma respectively was registered. The number of cells counted microscopically was found to be reduced to the same extent. This means that 79.4 and 44.1 per cent of the cells respectively detached during the culture period ($p<0.01$). The radioactivity found in the sediment (undigested Candida particles in detached cells during the digestion period from the 8th to the 9th day of culture) increased from 5.6 ± 1.2 (normal serum) to 14.3 ± 3.4 per cent (100 per cent uraemic plasma) ($p<0.01$)

The radioactivity on the coverslips (un-

plasma, but impaired phagocytic capacity in the presence of autologous predialysis plasma. Using monocytes from uraemic patients as macrophage precursors, *Urbanitz & Sieberth* (15) found a decreased engulfment of IgG coated red cells in the presence of normal serum.

Publications on the behaviour of human cells under uraemic conditions are few and the causes of the increased frequency of infections in uraemic patients are generally unknown. The aim of the present work was therefore, to investigate functional alterations in human mononuclear phagocytes caused by plasma from uraemic patients, using standardized test procedures.

MATERIALS AND METHODS

Venous blood was obtained from 18 uraemic patients with serum creatinine higher than 10 mg/100 ml.

Some patients were treated with haemodialysis, others were on dietary treatment. Only predialysis plasma was used from the dialysis patients. 0.05 mg heparin (Nyco A/S Norway) per ml was added to the samples. The blood cells were spun down, and if not used immediately the plasma was stored in deep frozen state. As control, pooled A serum from healthy adults was used, also with 0.05 mg heparin per ml added.

Cell Culture

The general methods for separation and culturing of human monocytes and for testing of phagocytosis have been reported in detail previously (16 18). Defibrinated venous blood from healthy adults was separated by gradient centrifugation using Lymphoprep (Nyco A/S Norway) as described by *Boyum* (2). After washing in Hanks balanced salt solution (BSS) the mononuclear cells were suspended in three parts of the medium RPMI 1640 (Flow Scotland) supplemented with one part of pooled human A serum, 0.1 mg l glutamin per ml and 40 µg gentamicin per ml (complete medium). The solution was dispensed in volumes of 0.5 ml with 3×10^6 cells per ml on to glass coverslips (Leighton type 11 × 35 mm) in petri dishes (Nuclon, Denmark). The cells were cultured in a National CO_2-incubator (USA) at 37 C with 5 per cent CO in air and with 100 per cent humidity. After an incubation period of 90 min, the medium containing non-adherent cell (lymphocytes) was removed and 2.5 ml of fresh complete medium was added to each culture dish. The medium was changed on the first and the fourth day of culture.

Candida albicans

Candida albicans (Department of Microbiology Regional Hospital University of Trondheim) were heat-killed at 60 C for 2 hours, and labelled with ^{125}I by means of electrolysis (17).

Test Procedure A

After washing the coverslips five times in Hanks BSS the cells were incubated with either 2.5 ml 100 per cent uraemic plasma, 2.5 ml uraemic plasma diluted to 25 per cent with RPMI 1640, 2.5 ml 100 per cent normal serum, or 2.5 ml 25 per cent normal serum diluted with RPMI 1640 from (a) 4th to the 8th day (b) 8th to the 10th day and c) 8th to the 12th day after start of cultura.

Uraemic plasma with 25 per cent normal serum was also used as culture medium from the 4th to the 8th day with normal serum as control.

At the end of the incubation period the media were removed and each petri dish was incubated for 15 min with 2.5 ml complete medium containing 2×10^6 I-labelled Candida particles per ml. The coverslips were then washed six times in complete medium and placed in new petri dishes, and fresh medium with 25 per cent normal pooled A-serum was added.

Mononuclear phagocytes were also exposed to equal amounts of uraemic serum/normal serum and normal plasma/normal serum from the 4th to the 8th day of culture as control experiments.

In some experiments, the numbber of cells on the coverslips was estimated microscopically using an inverted phase contrast microscope (Reichert, Austria) before the Candida particles were added.

The cultures were harvested after 24 hours. The medium was collected and centrifuged at 2000 G for 10 min. The radioactivity on each coverslip in the supernatant and in the sediment, was counted using a Wallac GM gamma radiation counter (Finland).

Test Procedure B

Mononuclear phagocytes were cultured in complete medium for eight days. The media were then removed and the cells were incubated with either 100 per cent uraemic plasma, 100 per cent normal serum, 25 per cent uraemic plasma or normal serum diluted in RPMI 1640. The media contained 2×10^6 ^{125}I labelled Candida particles per ml. After incubation for 15 min the coverslips were washed 12 times in Hanks BSS and the radioactivity was registered.

TABLE 4 *Effect of Two Different Concentrations of Uraemic Plasma and Normal Serum on the Digestion of Engulfed Radiolabelled* Candida albicans *in 8-Day-Old Human Mononuclear Phagocytes*

Test media	Total radioactivity per culture dish	Distribution of radioactivity in different culture compartments after the digestion period from the 8th to the 9th day		
	Per cent of controls	Coverslips per cent	Sediment per cent	Cell-free medium per cent
Uraemic plasma 100 per cent	101.9 ± 10.3	19.3 ± 5.1	12.5 ± 3.3	68.1 ± 6.5
Normal serum 100 per cent	100*	26.9 ± 5.1	4.2 ± 0.4	68.9 ± 4.9
Uraemic plasma 25 per cent	107.6 ± 7.2	21.1 ± 3.5	6.8 ± 1.0	72.1 ± 3.8
Normal serum 25 per cent	100‡	20.7 ± 3.6	4.8 ± 0.5	74.5 ± 3.9

* Range 33–243 × 10^3 ct/min radioactivity per cell dish.
‡ Range 22–259 × 10^3 ct/min radioactivity per cell dish.
The values given are the means ± S.E.M. of the results from 8 experiments.
Each experiment was performed in triplicate culture dishes.
The radioactivity in the culture compartments is expressed as percentage of the total radioactivity in each culture dish.

TABLE 5 *Phagocytosis of Radiolabelled* Candida albicans *in 8-Day-Old Human Mononuclear Phagocytes Cultured under Influence of Urea and Creatinine from the 4th to the 8th Day Before Test Start*

Concentrations	Total radioactivity per culture dish	Distribution of radioactivity in different culture compartments after the digestion period from the 8th to the 9th day		
	Per cent of controls	Coverslips per cent	Sediment per cent	Cell-free medium per cent
Urea 990 mg/100 ml	102.0 ± 4.1	22.8 ± 1.8	5.3 ± 1.1	72.1 ± 1.6
Creatinine 33 mg/100 ml	103.5 ± 2.5	22.6 ± 2.0	5.1 ± 0.8	72.3 ± 1.7
Urea 990 mg/100 ml and creatinine 33 mg/100 ml	98.2 ± 5.3	22.7 ± 1.9	5.2 ± 0.9	72.1 ± 1.7
Control	100*	22.3 ± 1.8	4.9 ± 0.7	72.9 ± 1.5

* Range 64–118 × 10^3 ct/min radioactivity per culture dish.
The values given are the means ± S.E.M. of the results from seven experiments.
Each experiment was performed in triplicate culture dishes.
The radioactivity in the culture compartments is expressed as percentage of the total radioactivity in each culture dish.

detachment of cells cultured in 100 per cent and 25 per cent uraemic plasma increased to 51.2 and 28.2 per cent respectively as compared to the controls.

The detachment during the digestion phase increased from 3.7 ± 0.5 (100 per cent normal serum) to 9.3 ± 4.2 per cent (100 per cent uraemic plasma) ($p > 0.05$) and from 3.7 ± 0.6 to 4.2 ± 0.5 per cent in 25 per cent normal serum and 25 per cent uraemic plasma, respectively ($p > 0.05$). The digestion capacity of the remaining cells decreased from 68.4 ± 2.7 to 52.8 ± 4.2 per cent (100 per cent normal serum compared with 100 per cent uraemic plasma) ($p < 0.01$) and from 69.7 ± 2.7 to 65.9 ± 3.0 per cent (25 per cent normal serum compared with 25 per cent uraemic plasma) ($p < 0.01$)

c) In normal *macrophages* cultured in uraemic plasma from the 8th to the 12th day the digestion capacity of the cells decreased even more viz. from 71.4 ± 2.2 (normal se

TABLE 2. *Phagocytosis of Radiolabelled* Candida albicans *in 10-Day-Old Human Mononuclear Phagocytes Cultured in Two Different Concentrations of Uraemic Plasma and Normal Serum from the 8th to the 10th Day before Start of Test*

Test media	Total radioactivity per culture dish	Distribution of radioactivity in different culture compartments after the digestion period from the 10th to the 11th day		
	Per cent of controls	Coverslips per cent	Sediment per cent	Cell-free medium per cent
Uraemic plasma 100 per cent	48 8 ± 16 9	38.0 ± 3.0	9.3 ± 4.2	52.8 ± 4.2
Normal serum 100 per cent	100*	28 0 ± 2 4	3 7 ± 0 5	68.4 ± 2.7
Uraemic plasma 25 per cent	71 8 ± 15 4	30 0 ± 2.8	4.2 ± 0.5	65.9 ± 3.0
Normal serum 25 per cent	100‡	26.0 ± 2.9	3 7 ± 0.6	69 7 ± 2.7

* Range 196–343 × 10³ ct/min radioactivity per culture dish.
‡ Range 167–393 × 10³ ct/min radioactivity per culture dish.
The values given are the means ± S.E.M of the results from 5 experiments.
Each experiment was performed in triplicate culture dishes.
The radioactivity in the culture compartments is expressed as percentage of the total radioactivity in each culture dish.

TABLE 3 *Phagocytosis of Radiolabelled* Candida albicans *in 12 Day-Old Human Mononuclear Phagocytes Cultured in Two Different Concentrations of Uraemic Plasma and Normal Serum from the 8th to the 12th Day before Start of Test*

Test media	Total radioactivity per culture dish	Distribution of radioactivity in different culture compartments after the digestion period from the 12th to the 13th day		
	Per cent of controls	Coverslips per cent	Sediment per cent	Cell-free medium per cent
Uraemic plasma 100 per cent	37.2 ± 10.5	41 0 ± 6.7	20 1 ± 5 8	44.5 ± 10.0
Normal serum 100 per cent	100*	24.5 ± 2.2	4 1 ± 0 6	71 4 ± 2.2
Uraemic plasma 25 per cent	46.9 ± 9 4	27 9 ± 2.5	9 0 ± 2.6	63.2 ± 4.0
Normal serum 25 per cent	100‡	24.0 ± 2 0	3 4 ± 0.2	72.3 ± 1.8

* Range 91–213 × 10³ ct/min radioactivity per culture dish.
‡ Range 132–239 × 10³ ct/min radioactivity per culture dish.
The values given are the means ± S.E.M. of the results from 6 experiments.
Each experiment was performed in triplicate culture dishes.
The radioactivity in the culture compartments is expressed as percentage of the total radioactivity in each culture dish.

digested Candida particles in macrophages remaining on the coverslips) increased from 26 4 ± 3.2 (normal serum) to 43.2 ± 6 4 per cent (100 per cent uraemic plasma) The radioactivity in the cell-free medium decreased from 68 0 ± 4 0 (normal serum) to 42.5 ± 7 3 per cent (100 per cent uraemic plasma) These findings indicate a decreased digestive ability of 23.5 per cent ($p<0.01$) in the cells pretreated with 100 per cent uraemic plasma. After pretreatment with 25 per cent uraemic plasma the digestive ability was reduced by 7 1 per cent ($p<0.01$)

When the cells were cultured in plasma as compared to serum, no difference in the cell morphology or function could be registered.

b) Table 2 shows the functional alterations of 8-day-old normal macrophages cultured in uraemic plasma from the 8th to the 10th day before start of the test. The

Test Procedure E

In order to examine the degree of lysis of mononuclear blood cells induced by 100 per cent uraemic plasma, the release of radioactivity from ^{51}Cr-labelled cells was measured. The mean values of eight experiments, expressed as percentage of the total radioactivity (supernatant and sediment) after 24 hours, were 26.2 ± 2.6 (uraemic plasma) and 26.2 ± 2.7 (controls). After 72 hours a slight but significant ($p<0.01$) increase from 38.5 ± 1.3 (uraemic plasma) to 41.2 ± 1.0 (controls) was seen.

DISCUSSION

Uraemic plasma was found to have a marked depressive effect on the macrophage function. The results from experiment to experiment showed some variation, partly due to variation in quality from cell culture to cell culture, but possibly also due to variation in concentrations and quality of possible inhibitory factor(s) in plasma from different uraemic donors. The results from the experiments where the cells were cultured in uraemic plasma at different intervals from the 4th to the 12th day after start of culture show that the detachment from the glass surface of the coverslips was less pronounced the more differentiated the cells were before exposure to uraemic plasma.

Further the depressive effect of uraemic plasma on the function of the macrophages was found to be more pronounced after an exposure period of four days as compared to two days, thus indicating a time-dependent relationship.

Both the adhesiveness of macrophages to surfaces and the attachment/engulfment and digestion of the Candida particles are dependent on a vital cell membrane and an intact cell metabolism. A diminished adhesiveness to glass surface reflects an impaired membrane function. An explanation may be that adhesiveness to glass surface depends on IgG receptors (11) and the engulfment of Candida particles is caused by a non-immunological surface receptor the latter not being influenced by uraemic plasma. Another explanation may be that uraemic plasma causes a decreased energy production generally in the cells essential for maintenance of a normal membrane function, thus resulting in detachment of some of the cells. Investigations of monocytes from uraemic patients (15) have revealed a decreased phagocytosis of IgG-coated red cells parallel to an enhanced spreading activity on glass surfaces. This is not necessarily conflicting, because the experiments were carried out on uraemic cells in normal medium.

It has been demonstrated that it is necessary to culture the macrophages for more than one day to get an impaired digestion capacity. By first loading the cells with Candida particles and then adding uraemic plasma, the digestion was normal after one day but three times as many cells had detached in 100 per cent uraemic plasma as compared to the control.

The formation of phagosomes and phagolysosomes depends on the completion of membrane fusion, and the digestion itself depends on the adequate formation of lysosomes. The digested material is released to the cytoplasma and transferred to the culture medium. Lysosomes which have completed their digestion may also fuse with the cell membrane and extrude their contents. The decreased digestive ability of the cells measured after two to four days pre-treatment with uraemic plasma indicates that one or some of these cellular functions are depressed.

The phagocytosis is an energy requiring activity and it is well-known that the cell metabolism is altered in uraemic patients. It has been shown that both the glucose utilization via the Krebs cycle and the uncoupling of oxidative phosphorylation (1?) are altered in uraemia. This may lead to a reduced production of ATP. The presence of ATP-ase on the macrophage membrane may also be important for the ingestion phase of phagocytosis (9). The engulfment can be inhibited by glycolytic inhibitors like iodoacetate (6). Preliminary investigations indicate that mono-

TABLE 6 *Phagocytosis of Radiolabelled* Candida albicans *in 8 Day-Old Human Blood Mononuclear Phagocytes Cultured under Different Concentrations of Methylguanidine from the 4th to the 8th Day before Start of Test*

Concentrations	n	Total radioactivity per culture dish	Distribution of radioactivity in different culture compartments after the digestion period from the 8th to the 9th day		
		Per cent of controls	Coverslips per cent	Sediment per cent	Cell-free medium per cent
1.2 mg/100 ml	5	98.5 ± 5.9	25.5 ± 2.4	5.8 ± 0.9	68.7 ± 3.0
Control		100*	26.7 ± 1.8	7.6 ± 1.8	65.0 ± 3.6
3.0 mg/100 ml	6	92.2 ± 6.1	25.4 ± 2.4	6.5 ± 1.5	68.2 ± 3.7
Control		100§	25.5 ± 2.3	7.0 ± 1.6	67.4 ± 3.6
6.0 mg/100 ml	11	88.0 ± 4.6	31.0 ± 1.9	4.9 ± 0.3	63.9 ± 2.0
Control		100‡	30.4 ± 1.8	4.7 ± 0.3	64.9 ± 1.9
12.0 mg/100 ml	9	75.3 ± 3.9	34.6 ± 2.1	5.7 ± 0.7	59.8 ± 2.2
Control		100†	31.0 ± 2.0	4.7 ± 0.4	64.3 ± 2.2
24.0 mg/100 ml	9	56.1 ± 8.1	37.3 ± 2.1	6.3 ± 0.9	57.1 ± 2.3
Control		100‡	31.0 ± 2.0	4.7 ± 0.4	64.3 ± 2.2

Range *108–260 × 10^3 §83–260 × 10^3 †69–268 × 10^3 ‡83–260 × 10^3 ct/min radioactivity per cultured dish.
The values given are the means ± S.E.M. of the results.
Each experiment was performed in triplicate culture dishes.
The radioactivity in the culture compartments is expressed as percentage of the total radioactivity in each culture dish.

rum) to 44.3 ± 10.0 (100 per cent uraemic plasma) ($p<0.01$) (Table 3)

d) Using a culture medium containing 75 per cent uraemic plasma and 25 per cent normal serum from the 4th to the 8th day of culture the results did not differ significantly ($p>0.05$) from those where only uraemic plasma was used.

Test Procedure B

After incubation for 15 min no significant immediate effect of uraemic plasma on the attachment/engulfment of ^{125}I labelled Candida particles was found. Mean values of the results of 10 experiments ± S.E.M. were 104.0 ± 9.0 in 100 per cent and 110.7 ± 7.1 in 25 per cent uraemic plasma as compared to the controls ($p>0.05$)

Test Procedure C

The presence of uraemic plasma had no influence on the digestion of Candida particles engulfed under normal conditions (Table 4) The decrease in radioactivity on the coverslips (26.9 ± 5.1 in normal serum compared with 19.3 ± 5.1 per cent in 100 per cent uraemic plasma) ($p<0.01$) and the increase in radioactivity in the sediment (4.2 ± 0.4 in normal serum compared with 12.5 ± 3.3 per cent in uraemic plasma) ($p<0.01$) reflect greater detachment of cells caused by uraemic plasma during the digestion period.

Test Procedure D

When the cells were cultured in the presence of urea (990 mg/100 ml) and/or creatinine (33 mg/100 ml) no effect on the attachment to the coverslips or the digestion capacity of the cells was found (Table 5) A dose-dependent increase in detachment of the cells was found when the cells were exposed to methylguanidine under the same culture conditions, a slight inhibition of the digestive ability of the remaining cells being registered (from 64.3 ± 2.2 to 57.1 ± 2.3 after exposure to 24 mg/100 ml) ($p<0.01$) (Table 6)

Evidence for depletion of ital substances in hemodialysis. Trans. Am. Soc. artif. internal Organs 17 96–101 1971

11 *Rabinovitch, M.* Macrophage spreading in vitro. In *van Furth R.* (Ed.): Mononuclear Phagocytes in immunity infection and pathology Blackwell Scientific Publication, Oxford 1975 pp 369–385

12. *Renner D & Heintz, R.* Metabolic changes in uremia, Proc. Fourth International Congress of Nephrology Stockholm 2 248–252, 1969

13 *Shainkin, R., Giatt G & Berlyne M* The presence and toxicity of guanidinopropionic acid in uremia. Kidney International 7 s-302–s-305, 1975.

14 *Sanderson A R.:* Application of no-immune cytolysis using radiolabelled target cells. Nature (Lond.) 204 250–253 1963

15 *Urbanitz, D & Sieberth H G.:* Impaired phagocytic activity of human monocytes in respect to reduced antibacterial resistance in uremia. Clinical Nephrology 4 1 13–17 1975

16 *Viken K. E. & Odegaard A.* Phagocytosis of heatkilled radiolabelled *Candida albicans* by human blood monocytes cultured in vitro Acta path. microbiol. scand. Sect. B, 82 235–244 1974

17 *Viken K E.* ^{125}I-labelling of *Candida albicans* by electrolysis. Acta path. microbiol. scand. Sect. B 82 219–222, 1974

18. *Odegaard A., Viken K. E. & Lamvik J O.* Structural and functional properties of blood monocytes cultured in vitro Acta path. microbiol. scand. Sect. B, 82 223–234 1974

cytes from uraemic patients cultured in normal medium behave like normal cells" as regards phagocytosis of Candida particles in our test system (unpublished) This is consistent with the results of *Burleson* (1), and indicates that uraemic plasma contains components which depress phagocytosis.

Urea and creatinine separately and together in much higher concentrations than those usually measured in serum from uraemic patients, did not change the macrophage function. This is in agreement with *Quadracci et al* (10) who found no significant effect on cell growth (fibroblasts) by urea in concentrations up to 300 mg/100 ml and creatinine up to 10 mg/100 ml. At extremely high concentrations we found that methylguanidine exerted a significant depressive effect on macrophage functions. However these were much higher concentrations than those measured in serum from uraemic patients (13)

The inhibitory effect on human mononuclear phagocytes cultured *in vitro* caused by uraemic plasma must be due to some factor(s) present in the plasma 25 per cent normal serum is usually required for normal cell growth. A possible explanation for the loss of adhesiveness and the depressed digestive capacity might be lack of components in uraemic plasma which are necessary for the cell growth. However when the cells were cultured in uraemic plasma supplemented with 25 per cent normal serum the cell functions were depressed to the same extent as in 100 per cent uraemic plasma. Furthermore, the toxicity became less pronounced when the uraemic plasma was diluted to 25 per cent The inhibition of the digestion was the same whether the macrophages were exposed to uraemic plasma in their differentiation period or as more mature macrophages.

The depressive effect on mononuclear phagocytes of possible factors in uraemic plasma may explain in part the increased frequency of inflammation conditions in patients suffering from renal failure.

The technical assistance of Mrs. *B Lippe* and Mrs. *A Remen* is gratefully acknowledged We are also indebted to Professor *J O Lamvik* and Dr *K J Berg* for assistance during the experimental work and the preparation of the manuscript. *S Jorstad* is a research fellow of the *County of Sør-Trøndelag* and *K E Viken* has been a research fellow of the *Norwegian Research Council for Science and the Humanities.*

The work was supported by grants from the *Norwegian Research Council for Science and the Humanities* the *Norwegian Cancer Society* and the *Norwegian Society for Fighting Cancer*

REFERENCES

1 *Burleson R L.* Reversibel inhibition of phagocytosis in anephric uremic patients. Surg. Forum *24* 75–77 1973
2 *Bøyum A* Separation of leucocytes from blood and bone marrow Scand. J Clin. Lab Invest. *21* Suppl 97 1968.
3 *van Furth R Langevoort H L & Schaberg A* Mononuclear phagocytes in human pathology-proposal for an approach to improved classification. In *van Furth, R.* (Ed.) Mononuclear phagocytes in immunity infection and pathology Blackwell Scientific Publication. Oxford, 1975 pp. 1–15
4 *Gombos E A Jefferson D M & Bird J G* Effect of haemodialysis on immune response. Proceedings of the European Dialysis and Transplant Association *11* 367 373, 1975
5 *Hanicki, Z Cichocki T Sarnecka-Keller M Klein A & Komorowska Z.* Influence of middle-sized molecule aggregates from dialysate of uremic patients on lymphocyte transformation *in vitro* Nephron *17* 77–80 1976
6 *Karnovsky M L. Simmons S., Glass E. L, Shafer A W & Hart P O* Metabolism of macrophages. In *van Furth* (Ed.) Mononuclear phagocytes. Blackwell Scientific Publication, Oxford 1970 pp. 103–120
7 *Newberry W M & Sandford J P* Defective cellular immunity in renal failure Depression of reactivity of lymphocytes to phytohemaglutinin by renal failure serum J clin. Invest. *6* 1262–1268, 1971
8. *Nguen Khoa M Terlain B Paris J Werner G Sausse A & Funk-Brentano J L.* An approach to "middle molecules identification in artificial kidney dialysate, with reference to neuropathy prevention. Trans Am Soc. artif internal Organs *19* 320-324 1973
9 *North R J* Endocytosis. Sem. Hemat *7* 161–171 1970
10. *Quadracci L Gambi I Christopher T Harker L & Striker G* Assay of serum abnormalities in uremia and dialysis patients

consistent with low IgA levels in serum and lack of IgA plasma cells in the tonsils (*Ostergaard* 1976).

PATIENTS AND METHODS

The series comprises 27 children, 6 to 11 years of age, who 2 years and 6 months earlier had their tonsils excised because of recurrent and chronic tonsillitis. Originally the series comprised a group of 28 children, who like 24 older children had been tonsillectomized; the series has been published elsewhere (*Ostergaard* 1975, *Ostergaard* 1976). In contrast to findings in the older patients, the levels of IgA in serum and saliva had been rather low in the younger children prior to the performance of tonsillectomy. At the medical check up 2 years and 6 months after tonsillectomy all the 27 young patients (or their parents) were specifically questioned about the frequency of which infections had occurred during the intervening period of time. All the children had their throats inspected and a swab from the empty tonsillar beds was taken for bacterial culture.

Controls. 27 healthy controls who matched the patients with regard to sex and age were selected. None of the controls had experienced recurrent and chronic infections and they had never been hospitalized. None of the controls had previously been subjected to adenoidectomy or tonsillectomy. The controls in the present study were not the same as those participating in an earlier study of the same patients.

Samples of serum and saliva were collected from all the patients and controls; the samples were frozen immediately at −20° C until determination of immunoglobulins. The saliva was always tested for blood contamination, using haemastix, and found to be negative. Signs of inflammation of the mouth were not observed in either of the patients or controls.

Serum and saliva IgG-, IgA- and IgM assays were performed by the Department of Clinical Chemistry, Aalborg Hospital, South. Rabbit-anti-human-IgG-, IgA- and IgM obtained from Dacopatts, Copenhagen, were used. Details concerning the methods used are presented elsewhere (*Ostergaard* 1976). Pooled human serum, calibrated in terms of the WHO reference standard preparation (*Rowe et al.* 1972), was used as a standard in estimations of serum as well as of saliva immunoglobulins. Furthermore, the serum standard was the same as that used in earlier studies of the same patient. The pre- and postoperative variation of the serum standard was less than 3 per cent. The day precision of the runs (= 2 days) was 6.5 per cent, 6.3 per cent and 5.5 per cent for IgG, IgA and IgM respectively. The sensitivity of detection of saliva immunoglobulins was 0.09, 0.34 and 2.04 IU/ml for IgG, IgA and IgM, respectively.

Serum IgE was estimated by the Department of Clinical Chemistry, Odense University Hospital. A radio-immuno-sorbent Test (Phadebas kit, Pharmacia, Copenhagen) was used. The sensitivity of detection of IgE in serum was 10 U/ml.

Bacterial cultures were performed by the Department of Medical Microbiology, Aalborg Hospital, South. Less than half an hour after the swab was taken, it was spread onto blood agar and chocolate agar plates. Incubation was at 35° C, aerobically and anaerobically, and the plates were read after 24 and 48 hours. Details concerning the characteristics used for identification of the various pathogens are published elsewhere (*Ostergaard* 1976).

The results of the *immunofluorescent studies* of the patients shortly after tonsillectomy have been published earlier (*Ostergaard* 1975). The results are expressed as − (no immunofluorescent positive cells), +(−) (occasionally a single cell), + (2–4 cells), ++ (5–10 cells), +++ (10–20 cells) and ++++ (more than 20 cells) in sections of approximately 5 × 5 mm.

As regards the unpaired observations, the results were performed by the Mann-Whitney U test. In the case of paired observations, the Wilcoxon test was used. P values, <0.05 were considered significant.

RESULTS

Examination of the patients. Twenty two of the patients (or their parents) declared that tonsillectomy had resulted in a considerable decreased tendency to develop recurrent infections of the throat. The remaining 5 patients had still complaints of recurring respiratory infections, in 4 of the latter of recurring and chronic otitis media (patients nos. 1, 3 and 8, Table 3, and patient no. 16, Table 4). One patient had repeated attacks of pharyngitis (patient no. 26, Table 5) and a considerable lymphatic hyperplasia of the pharyngeal mucosa. In addition, this patient had a typical history of febris aestivalis. Furthermore one patient had a confirmed history of bronchial asthma before tonsillectomy was performed (patient no. 27, Table 5). In the intervening period of time, atopic diseases had developed in a further 3 children, bronchial asthma had developed in two, atopic dermatitis in one (patients nos. 3 and

Acta path microbiol. scand Sect C 85 178–186 1977

IgA LEVELS AND CARRIER RATE OF PATHOGENIC BACTERIA IN 27 CHILDREN PREVIOUSLY TONSILLECTOMIZED

POUL AABEL ØSTERGAARD

Otological Department, Aalborg Hospital North Department of Clinical Chemistry and Department of Medical Microbiology (Statens Seruminstitut) Aalborg Hospital South, Aalborg Denmark

Østergaard, P Aa. IgA levels and carrier rate of pathogenic bacteria in 27 children previously tonsillectomized Acta path. microbiol scand. Sect. C, 85 178– 186 1977

The object of the present paper is to present laboratory and clinical data on 27 children of ages between 6 and 11 years who in connection with tosillectomy 2½ years earlier had been found to have low serum and saliva IgA levels, low serum IgE levels, and a considerable lack of IgA and IgE plasma cells in the excised tonsils correlation between deficiency in IgA and culture of pathogenic bacteria from the tonsils was significant. From a clinical point of view 22 of the children had benefit of the tonsillectomy and had no longer a tendency towards a development of recurrent infections. The remaining 5 patients continued to complain of recurring respiratory infections In addition, levels of serum and saliva IgA were low Further more 4 of these 5 children harboured pathogenic bacteria in their throats. Many of the 2 patients still had low serum IgA and IgE levels as compared with levels in healthy age-related controls in 3 patients, however the IgE levels in serum had risen considerably parallel with the development of atopic diseases. Saliva IgA was rather constant after tonsillectomy as compared with the preoperative levels, though it had risen in some of the children. As regards serum IgG and IgM these immunoglobulins had decreased significantly and the question is raised, whether it might had been due to the tonsillectomy either by the removal of chronically infected organs or by the removal of important immunological tissue

Key words IgA levels carrier rate pathogenic bacteria previous tonsillectomy

P Aabel Østergaard Department of Paediatrics, Aalborg Hospital North, DK 9000 Aalborg Denmark.

Received 11 xi.76 Accepted 6 xi 76

Agammaglobulinaemia in connection with repeated and chronic infections has been investigated for more than twenty years. In recent years, the question has been raised whether a relative absence of immunoglobulins also is correlated with a tendency in children to develop recurring and chronic infections (*Donovan & Soothill* 1973 *Kuo et al* 1975)

The aim of the present study was to investigate the immunoglobulin formation in serum and saliva and the carrier rate of pathogenic bacteria in 27 children, who 2 years and 6 months earlier had been tosillectomized. At the time of tonsillectomy the lack of IgA and IgE plasma cells in the tonsils excised from these children was considerable (*Østergaard* 1975) and, in addition culture of pathogenic bacteria from their tonsils was

consistent with low IgA levels in serum and lack of IgA plasma cells in the tonsils (*Østergaard* 1976).

PATIENTS AND METHODS

The series comprises 27 children, 6 to 11 years of age, who 2 years and 6 months earlier had their tonsils excised because of recurrent and chronic tonsillitis. Originally, the series comprised a group of 78 children, who like 24 older children had been tonsillectomized; the series has been published elsewhere (*Østergaard* 1975, *Østergaard* 1976). In contrast to findings in the older patients, the levels of IgA in serum and saliva had been rather low in the younger children prior to the performance of tonsillectomy. At the medical check up 2 years and 6 months after tonsillectomy, all the 27 young patients (or their parents) were specifically questioned about the frequency of which infections had occurred during the intervening period of time. All the children had their throats inspected, and a swab from the empty tonsillar beds was taken for bacterial culture.

Controls. 27 healthy controls who matched the patients with regard to sex and age were selected. None of the controls had experienced recurrent and chronic infections and they had never been hospitalized. None of the controls had previously been subjected to adenoidectomy or tonsillectomy. The controls in the present study were not the same as those participating in an earlier study of the same patients.

Samples of serum and saliva were collected from all the patients and controls; the samples were frozen immediately at −20 C until determination of immunoglobulins. The saliva was always tested for blood contamination, using haemastix, and found to be negative. Signs of inflammation of the mouth were not observed in either of the patients or controls.

Serum and saliva IgG-, IgA- and IgM assays were performed by the Department of Clinical Chemistry, Aalborg Hospital South. Rabbit-anti-human-IgG-, IgA- and IgM, obtained from Dacopatts, Copenhagen, were used. Details concerning the methods used are presented elsewhere (*Østergaard* 1976). Pooled human serum, calibrated in terms of the WHO reference standard preparation (*Rowe et al.* 1972) was used as a standard in estimations of serum as well as of saliva immunoglobulins. Furthermore, the serum standard was the same as that used in earlier studies of the same patients. The pre- and postoperative variation of the serum standard was less than 5 per cent. The day precision of the runs ($n = 2$ days) was 6.5 per cent, 6.3 per cent and 5.5 per cent for IgG, IgA and IgM, respectively. The sensitivity of detection of saliva immunoglobulins was 0.09, 0.34 and 2.04 IU/ml for IgG, IgA and IgM, respectively.

Serum IgE was estimated by the Department of Clinical Chemistry, Odense University Hospital. A radio-immuno-sorbent Test (Phadebas kit, Pharmacia, Copenhagen) was used. The sensitivity of detection of IgE in serum was 10 U/ml.

Bacterial cultures were performed by the Department of Medical Microbiology, Aalborg Hospital, South. Less than half an hour after the swab was taken, it was spread onto blood agar and chocolate agar plates. Incubation was at 35 C, aerobically and anaerobically, and the plates were read after 24 and 48 hours. Details concerning the characteristics used for identification of the various pathogens are published elsewhere (*Østergaard* 1976).

The results of the *immunofluorescent studies* of the patients shortly after tonsillectomy have been published earlier (*Østergaard* 1975). The results are expressed as: − (no immunofluorescent positive cells), +(—) (occasionally, single cell), + (2–4 cells), ++ (5–10 cells), +++ (10–20 cells) and ++++ (more than 20 cells) in sections of approximately 5 × 5 mm.

As regards the unpaired observations, the results were performed by the Mann-Whitney U test. In the case of paired observations, the Wilcoxon test was used. P values, <0.05 were considered significant.

RESULTS

Examination of the patients. Twenty two of the patients (or their parents) declared that tonsillectomy had resulted in a considerable decreased tendency to develop recurrent infections of the throat. The remaining 5 patients had still complaints of recurring respiratory infections, in 4 of the latter of recurring and chronic otitis media (patients nos. 1, 5 and 8, Table 3, and patient no. 16, Table 4). One patient had repeated attacks of pharyngitis (patient no. 26, Table 5) and a considerable lymphatic hyperplasia of the pharyngeal mucosa; in addition, this patient had a typical history of febris aestivalis. Furthermore, one patient had a confirmed history of bronchial asthma before tonsillectomy was performed (patient no. 27, Table 5). In the intervening period of time, atopic diseases had developed in a further 3 children, bronchial asthma had developed in two, atopic dermatitis in one (patients nos. 3 and

TABLE 1 *Serum Ig-levels in Patients and Controls in 1973 and 1976*

	Serum Ig levels IU/ml							
	Patients				Controls			
	IgG	IgA	IgM	IgE	IgG	IgA	IgM	IgE
1973								
Mean	115	59.5	112	31.5	139	101	124	52
Range	*70–194*	*21–123*	*37–242*	*37–907*	*96–214*	*45–156*	*42 192*	*10–2[illegible]*
1976								
Mean	101	51	90	39	145	106	135	5[illegible]
Range	*69–149*	*17–114*	*36–167*	*17–1200*	*98–205*	*45–181*	*38–201*	*10–1[illegible]*

and 4 Table 3 and patient no 16 Table 4) These 3 children belonged in the group of 5 patients, in whom complaints of recurring respiratory infections persisted.

Serum immunoglobulin levels in the patients before and after tonsillectomy (1973 and 1976) and levels in their former and present controls are listed in Table 1 Serum IgA which in 1973 had been low in many of the patients (mean (median) 59.5 IU/ml)) had further decreased in 1976 (mean 51 IU/ml) the difference between the pre- and post operative levels was sigificant ($p<0.001$) As regards serum IgG and IgM the preoperative levels (mean 115 and 112 IU/ml respectively) did not differ significantly from the levels in their former controls (mean 139 and 124 IU/ml, respectively) Conversely IgG and IgM had declined 2 years and 6 months later (mean 101 and 90 IU/ml) the difference from the pre-operative levels was significant ($p<0.001$ and <0.0025 respectively) By 1976 the pre-operative levels of serum IgE which was significantly reduced in the patients had risen considerably in some of the children but the difference between pre and postoperative levels (mean 31.5 and 39 U/ml) was not significant ($p>0.25$)

As regards the serum immunoglobulin levels in the patients and their present controls (Table 1) the mean serum IgA (51 IU/ml) was still considerably different from a mean serum IgA of 106 IU/ml in the age-related controls ($p<0.001$) In addition, the postoperative mean levels of serum IgG and IgM (101 and 90 IU/ml) in the patients were different from the mean IgG and IgM levels in their present controls (145 and 135 IU/ml) ($p<0.001$ and <0.0025 respectively) In the case of IgE, the postoperative mean (39 U/ml) was significantly reduced as compared with the mean IgE levels in the present controls (mean 59 U/ml) ($p<0.025$)

TABLE 2 *Saliva IgA Levels in Patients and Controls in 1973 and 1976*

	Saliva IgA levels IU/ml	
	Patients	Controls
1973		
Mean	3.3	4.2
Range	1.8–7.9	1.9–8.4
1976		
Mean	3.4	3.9
Range	1.6–8.5	1.8–7.5

Saliva IgA levels are listed in Table 2. This immunoglobulin was demonstrated in all of the patients before as well as after tonsillectomy Despite an increase in saliva IgA was observed in some of the patients, there was no significant difference between the pre- and postoperative means (3.3 and 3.4 IU/ml) ($p>0.1$) Mean saliva IgA was significantly reduced before tonsillectomy Conversely the postoperative mean saliva IgA in the patients (3.4 IU/ml) did not differ from a mean saliva IgA of 3.9 IU/ml in the present controls ($p>0.05$)

TABLE 3 *Correlation between IgA and IgE Levels in 1973 and 1976 and the Results of the Immunofluorescent Studies on IgA and IgE Plasma Cells in Patients Carrying* Haemophilus influenzae *in 1973*

Patient number	Immunofluorescent studies 1973		Mean serum Ig-levels IU/ml				Mean saliva IgA IU/ml		Bacterial culture 1976
			IgA		IgE				
	IgA	IgE	1973	1976	1973	1976	1973	1976	
1	+ (—)	—	51	51	48	32	2.1	2.3	Pneum.
2	—	—	31	65	14	11	4.5	5.7	NBF
3	—	—	43	41	34	1030	2.0	1.4	Pneum.
4	—	+	50	44	27	1200	1.9	1.4	NBF
5	+ +	—	116	65	57	34	5.8	3.0	NBF
6	+	—	60	61	26	19	3.3	3.4	NBF
7	—	—	40	17	43	32	2.5	4.9	NBF
8	—	—	41	38	26	32	2.4	1.4	Pneum.
9	—	—	21	23	13	11	2.4	4.4	NBF
Mean			43	41	27	32	2.4	3.0	

NBF = Normal Bacterial Flora.
Pneum = Pneumococci.

TABLE 4 *Correlation between IgA and IgE Levels in 1973 and 1976 and the Results of the Immunofluorescent Studies of IgA and IgE Plasma Cells in Patient who in 1973 had been Carrying Beta-haemolytic Streptococci in 1973*

Patient number	Immunofluorescent studies 1973		Mean serum Ig-levels IU/ml				Mean saliva IgA IU/ml		Bacterial culture 1976
			IgA		IgE				
	IgA	IgE	1973	1976	1973	1976	1973	1976	
10	—	—	41	55	32	21	4.0	2.5	NBF
11	+ +	+	96	114	48	44	7.8	7.3	NBF
12	+ + +	—	79	59	21	32	7.4	7.8	NBF
13	—	—	40	41	268	186	5.4	8.5	NBF
14	+ + +	+	50	41	196	122	2.2	2.9	NBF
15	+	—	66	75	27	19	1.1	1.6	NBF
16	—	—	41	35	27	280	2.9	1.4	H. infl.
17	+ +	—	93	72	21	14	7.9	7.1	NBF
18	+ +	—	88	41	19	11	4.2	5.9	NBF
19	+ +		50		60		1.8		
20	—	—	36	19	33	60	3.6	3.4	NBF
Mean			50	43	32	44	3.6	4.7	

NBF = Normal Bacterial Flora.
H infl = *Haemophilus influenzae*

As regards saliva IgG this immunoglobulin was demonstrated in 25 of the patients prior to tonsillectomy in 1976, however saliva IgG was only detectable in 14 of the patients the difference between the pre- and postoperative levels was found to be significant ($p<0.01$) Saliva IgM was not demonstrated in the patients before tonsillectomy but 2 years and 6 months later it was found in 3 patients, in whom saliva IgA levels were high (patient no. 9 Table 3 and patients nos. 11 and 12, Table 4)

TABLE 5 *Correlation between IgA and IgE levels in 19 3 and 19/6 and the Results of the Immunofluorescent Studies of IgA and IgE Plasma Cells in Patients Carrying Normal Bacterial Flora and Staphylococcus aureus in 1973*

Patient number	Immunofluorescent studies 1973		Mean serum Ig-levels IU/ml				Mean saliva IgA IU/ml		Bacterial culture 1976
			IgA		IgE				
	IgA	IgE	1973	1976	1973	1976	1973	1976	
21	+	+	45	44	31	27	3 6	2.9	NBF
22	+ +	—	75	56	32	29	2.2	3.7	NBF
23	+ +	+	59	23	27	23	4.2	1 4	NBF
24	+ + +	+	76	75	32	38	3.8	3 4	NBF
25	—	+	123	79	16	11	3 0	3 7	NBF
26	+ + +	+ +	86	79	907	640	3 4	3 4	NBF
27	+	—	88	59	696	440	2.3	1.6	NBF
28	+ +	+ +	58	48	31	43	5 4	5 4	NBF
Mean			75.5	57.5	32	38	3.5	3.4	

NBF = Normal Bacterial Flora.

TABLE 6 *Comparison between Levels of Serum and Saliva IgA in Patients in 1973 and 1976 with Regard to Negative or Positive IgA Plasma Cell Fluorescence of Tonsil Tissue*

	Mean serum IgA IU/ml with negative IgA fluorescence		Mean serum IgA IU/ml with positive IgA fluorescence	Mean saliva IgA IU/ml with negative IgA fluorescence		Mean saliva IgA IU/ml with positive IgA fluorescence
1973						
Mean	41		75.5	2.5		3 8
P =		<0 01			<0 02	
1976						
Mean	44 5		65	3.3		3.4
P =		<0 02			<0 1	

Results of the bacterial cultures All of the 22 patients who after the operation had no complaints of recurring respiratory infections harboured normal bacterial flora in their throats. Among the remaining 5 patients in whom respiratory infections were frequent 3 harboured pneumococci and 1 *Haemophilus influenzae* (non-capsular strain) In these 5 children, IgA as well as IgE plasma cells were lacking in the excised tonsils serum IgA and IgE as well as saliva IgA levels were low in all of these (patients nos. 1 3 8 and 9 Table 3 and patient no 16 Table 4) In addition, all 5 patients belonged to the group of children who prior to surgery had been harbouring pathogenic bacteria in the tonsils.

Tables 3 4 and 5 also show the correlation between results obtained earlier immunofluorescent studies of excised tonsils, of the pre and postoperative IgA and IgE levels in patients, and the results of bacterial cultures prior to and 2 years and 6 months after tonsillectomy It appears from Table 3 that the lack of IgA and IgE plasma cells in carriers of *Haemophilus influenzae* was considerable at the time of tonsillectomy It was observed in this particular group of patients that serum IgA as well as serum IgE levels and saliva IgA levels would be low prior to as

well as after tonsillectomy In addition, three of the patients in this group continued to harbour pathogenic bacteria in their throats 2 years and 6 months after tonsillectomy Table 4 shows that only one patient in the group of children who prior to tonsillectomy had harboured beta haemolytic streptococci in the tonsils, continued to harbour pathogenic bacteria in the throat 2 years and 6 months after tonsillectomy (*Haemophilus in fluenzae* patient no 16) In this group of patients, tonsils excised from 4 patients lacked IgA and 8 lacked IgE plasma cells in the ex cised tonsils. Furthermore, levels of serum IgA in these patients was generally low 2 years and 6 months later it had decreased the difference between the pre- and postoper ative levels was significant ($p<0.05$) Serum IgE levels in this group of patients remained generally unaltered ($p>0.05$) Conversely saliva IgA had risen, the difference being significant ($p<0.05$) the increase observed may have been due to a considerable rise to occur only in one patient (patient no. 13)

Table 5 shows the patients whose tonsils prior to tonsillectomy had harboured normal bacterial flora and *Staphylococcus aureus* These patients are treated as one group since the excised tonsils showed an almost normal occurrence of IgA and IgE plasma cells and relatively high levels of serum and saliva IgA prior to tonsillectomy In this group of patients, Serum IgA declined also 2 years and 6 months later the difference between the pre and postoperative levels was significant ($p<0.01$) Conversely serum IgE in this group of patients had increased a little but the increase was insignificant ($p>0.1$) The pre and postoperative saliva IgA levels in these patients remained unaltered ($p>0.01$)

Table 6 show the relationship between the pre and postoperative serum and saliva IgA levels in the patients with regard to the absence or presence of IgA plasma cells in their removed tonsils. A comparison between the pre and postoperative serum IgA levels in patient with and without positive IgA plasma cell fluorescence revealed a significant difference ($p<0.01$ and <0.02 respectively)

In addition, a comparison of the pre-opera tive saliva IgA levels and the absence or pre sence of IgA plasma cells had been found to differ significantly ($p<0.0?$) The same re sult was not obtained by comparison of post operative saliva IgA levels and absence or presence of IgA plasma cells ($p>0.1$)

Correlation between the pre and post operative IgE levels and absence or presence of IgE plasma cells was not observed.

DISCUSSION

It was of interest to note that levels of IgA in serum continued to be low in patients 2 years and 6 months after tonsillectomy furthermore, levels of this Immunoglobulin had declined as compared with the preoper ative levels. In addition, the decrease in levels of serum IgG and IgM was significant. Serum IgE levels which prior to tonsillectomy had been low in most of the patients, re mained generally unaltered after tonsillec tomy in 3 patients, however IgE had increased considerably parallel with the devel opment of atopic diseases, which is in agree ment with the hypothesis that IgA deficiency is involved in the pathogenesis of atopy (*Taylor et al.* 1973)

Apparently levels of saliva IgA had increased in the patients, but the observed rise in mean saliva IgA was probably due to a considerable increase in a few patients. On the other hand, the slightly rise in levels of saliva IgA was consistent with the observa tion that most of the children had benefited from the operation in that they had no longer a tendency towards a development of sore throats which is in agreement with the sug gestion (*Bellanti* 1965) that local IgA anti bodies are more important for respiratory infections than circulating IgA antibodies. Fur thermore, it has been proposed that serum and secretory IgA originate from different lymphatic tissue (*Collins-Williams et al* 1972) On the other hand, the decreased tendency observed in most of the children to develop sore throats after tonsillectomy is in

agreement with other observations in children that tonsillectomy normally decrease the tendency towards development of recurrent sore throats (*Banham* 1968, *Evans* 1968, *Manson et al* 1968).

One problem with regard to the influence of secretory IgA on the defence of mucous membranes against infections is to define the borderline between normal and decreased levels of saliva IgA which, owing to a probable influence on saliva immunoglobulin concentrations of saliva flow rate, may be difficult. Another problem is the rather divergent normal saliva IgA values published (*Immonen* 1967, *Buckley et al* 1968, *South et al* 1968, *Berg & Johansson* 1969). The technique used in this study seemed to be rather reliable and sensitive (*Østergaard & Blom*), but although no direct stimulation was used during collection individual dilution of saliva is probably unavoidable.

It applies to almost all of the patients that the lack of IgA plasma cells in the earlier removed tonsils was consistent with low serum IgA before as well as after tonsillectomy. In the absence of adequate controls, the IgA and IgE deficiency as suggested by the immunofluorescent studies of tonsil tissue (*Østergaard* 1975) should therefore be taken with some reservation. A compensatory increase in IgM was not observed either in the tonsils or in serum or saliva. *Savilahti* (1973) defined an IgA deficiency state in cases where IgA were replaced by IgM in the mucous membranes and secretions. Considered from that point of view the patients concerned were not deficient in IgA. However, low levels, but not absence of IgA in serum and secretions in connection with recurrent and chronic infections have recently been reported (*Donovan & Soothill* 1973, *Buser et al.* 1974, *Kuo et al.* 1975).

A carrier rate of presumably pathogenic bacteria of approximately 15 per cent in the patients concerned is not very far from a carrier rate of such bacteria in 3 and 12 per cent among healthy school children (*Masters et al* 1958, *Cornfield & Hubbard* 1961, *Turk* 1963). It is remarkable, however, that the bacteria were found in 4 out of 5 children in whom complaints of recurring infections persisted 2 years and 6 months after tonsillectomy, whereas the remaining 22 patients without such complaints only carried normal bacterial flora in their throats. Furthermore, all the 5 patients concerned lacked IgA as well as IgE plasma cells in the excised tonsils and levels of IgA in serum and saliva were low. In addition atopic diseases had developed in two of the latter in the intervening period of time.

From an immunological point of view the patients had apparently not benefited from the operation, as the immunoglobulin levels were found to be slightly reduced in most of these. These findings are compatible with the view that antigens to invade the organism by the oral or nasal route give rise to antibody production in the lymphatic tissue of the pharynx, and that the immunocompetent cells circulate and reach the remaining lymphoid pool (*Hall et al* 1967, *Saito* 1967, *Surjan & Surjan* 1970, 1971). However, the observed decline in serum immunoglobulins in these children may also be due to the removal of chronically infected organs known to maintain antigen stimulation. *Veltri et al* (1972) studied 17 tonsillectomized children and demonstrated that serum IgG declined 12 months after tonsillectomy. They suggested that it was due to the fact that pathogenic bacteria had been harboured prior to tonsillectomy. In this series, however, IgG levels only declined to levels within the limits normally applying to age-related controls. In the present study, IgG as well as IgA and IgM in serum declined significantly as compared with levels in age-related controls. Therefore, the influence of tonsillectomy on the serum immunoglobulin levels may not be unmasked until several years after tonsillectomy.

An interrelation between IgA and IgE in the protection of mucous membrane has apparently been confirmed (*Stites et al* 1975) or disproven (*Polmar et al* 1975), thus leaving this problem unsolved. The presence of T lymphocytes has been shown to be important for the initiation of IgA antibody re-

sponses (*Clough et al.* 1971) and, based on these findings, *Lawton et al.* (1972) suggested that the inability of IgA bearing lymphocytes to undergo terminal differentiation to IgA producing plasma cells in IgA deficient co-individuals may be due to a defective co-operative interaction with T-lymphocytes. Furthermore *Tada et al.* (1972) have shown that T-lymphocytes are of importance for the regulation of the IgE antibody responses. Based on these observations, the combined IgA and IgE deficiency observed in some of the patients here discussed may have been the result of a reduced function of such T-cell "helper" function.

The investigations were supported by *Det Lægevidenskabelige Forskningsfond for Nordjyllands Amt*

REFERENCES

Benham T M A survey of over 1,000 school leavers who have their tonsils removed. J Laryng. 82 203–217 1968

Bellanti J A Artenstein M S & Buescher E L Characterization of virus neutralizing antibodies in human serum and nasal secretion. J Immunol. 94 344 352, 1965

Berg T & Johansson, S G Immunoglobulin levels during childhood with special regard to IgE. Acta Paediat. Scand. 58 513–525 1969

Buckley R. H Dees S C & O'Fallon W M Serum immunoglobulins II. Levels in children subjects to recurrent respiratory infections. Pediatrics 42 50–60, 1968.

Buser F Pfl giharajt R. & S hertz, R. Infektanfalligkeit und IgA Mangel beim Kleinkind. Quantitativ Untersuchung. Helv Paediat. Acta, Suppl. 32 20 1974

Clough J D Mims L H & Strober W Deficient IgA antibody to arsanilic acid bovine serum albumin (BSA) in neonatally thymectomized rabbits J Immunol. 106 1019–1025 1971

Collins-Williams C Kokubo H L Lamenza C., Nizami, R Chi A H Lewis-McKinley C., Comerford T A & Large, E A Incidence of isolated deficiency of IgA in the serum of Canadian children. Ann. Allerg 30 11 19 1972.

Cornfield D & Hubbard J P A four-year study of the occurrence of beta-hæmolytic streptococci in 64 school children. New Engl. J Med. 264 211 215 1961.

Donovan, R & Soothill, J F. Immunological studies in children undergoing tonsillectomy Clin. exp. Immunol. 14 347–357 1973

Evans H E Tonsillectomy and adenoidectomy. Review of published evidence for and against T and A. Clin. Pediat. 7 71 75 1968.

Hall J O Bede Morris B. V Sc Gisbane D Moreno R. S & Bessis M C. The ultrastructure and function of the cells in lymph following antigenic stimulation. J exp. Med. 125 91–109, 1967

Ismonen P. Levels of serum immunoglobulins IgA, IgG and IgM in the malabsorption syndrome in children. Ann. Paediat. Fenn. 48 212 218, 1967

Kuo K N., LLoyd-Roberts, G C Orme I M & Soothill J F Immunodeficiency and infantile bone and joint infection. Arch. Dis. Childh. 50 51–56, 1975.

Lawton A. R., Royal, S A., Self K. S & Cooper M D IgA determinants on B-lymphocytes in patients with deficiency in circulating IgA. J Lab. Clin. Med. 80 26–34 1972.

Mawson S R., Adlington P & Evans M A controlled study evaluation of adeno-tonsillectomy in children. J Laryng. 82 963–979 1968.

Masters P L., Brumfitt W & Mendez, R. L. Bacterial flora of the upper respiratory tract in Paddington families. Brit. Med J 1 1200–1205 1958

Polmar S H., Waldman T A & Terry W D. The relationship of IgA and IgE deficiency Birth Defects Orig. Art. Ser 11 147–150, 1975.

Rowe D S Anderson S G & Grab B. A research standard for human serum immunoglobulins, IgG IgA and IgM. Bull. Wld. Hlth. Org. 42 535–538, 1972.

Sato H Immunological functions of palatine tonsils. J oto-rhin.-laryng. Soc. Jap. 70 1303–1311 1967

Savilahti, E. IgA deficiency in children. Immunoglobulin-containing cells in the intestinal mucosa, immunoglobulins in secretions and serum IgA levels. Clin. exp. Immunol. 13 395–406, 1973

South M A., Cooper M D Wollheim F A. & Good R. A. The clinical significance of IgA deficiency. Studies in patients with agammaglobulinemia and ataxia-telangiectasia. Am. J Med. 44 168–178, 1968.

Stites D P., Ishizaka K & Fudenberg, H H. The relationship of IgA and IgE deficiency Birth Defects Orig Art. Ser 11 151 153 1975

Surjan, L. & Surjan M The antibody production of tonsils. Arch. klin. exp. Ohr.-Nas.-u. Kehlk. Heilk. 195 331 337 1970.

Surjan L. & Surjan M Immunological role of

human tonsils. Acta Otolaryng *71* 190–193 1971

Tada T Okumura, K & Taniguchi M Regulation of homocytotropic antibody formation in the rat, VII Carrier functions in the anti hapten homocytotropic antibody response, J Immunol. *106* 1012–1017 1972.

Taylor B., Norman A Orgel H Stokes C Turner M & Soothill J F Transient IgA deficiency and pathogenesis of infantile atopy Lancet *II* 111–114 1973

Turk D C Naso-pharyngeal carriage of haemophilus type b. J Hyg Camb. *61* 247–256 1963

Veltri R W., Sprinkle P M Keller S A & Chicklo J M Immunological changes in a pediatric otolaryngic sample subsequent to T & A. J Laryng Otol. *86* 905–916, 1972.

Østergaard P Aa. & Blom M., Whole saliva immunoglobulin levels in 60 healthy children Determined by a sensitive electroimmune technique after prior carbamylation (unpublished results)

Østergaard P Aa Presence of immunoglobulin-containing plasma cells in tonsils from tonsillectomized children. Acta path. microbiol. scand Sect. C, *83* 406–412, 1975.

Østergaard P Aa., IgA levels and carrier rate of *Haemophilus influenzae* and beta-haemolytic streptococci in children undergoing tonsillectomy Acta path. microbiol. scand. Sect. C, *84 290–298 1976*

Acta path. microbiol. scand. Sect. C, 85 187–195, 1977

IgA LEVELS, BACTERIAL CARRIER RATE, AND THE DEVELOPMENT OF BRONCHIAL ASTHMA IN CHILDREN

POUL AABEL ØSTERGAARD

Department of Pediatrics and Pathological Institute, Aalborg Hospital, North.
Department of Clinical Chemistry and Department of Medical Microbiology
(Statens Seruminstitut) Aalborg Hospital, South, DK 9000 Aalborg, Denmark

Østergaard, P. Aa. IgA levels, bacterial carrier rate, and the development of bronchial asthma in children. Acta path. microbiol. scand. Sect. C, 85 187–195, 1977

A total of 54 children, earlier hospitalized for asthma, were reinvestigated with regard to immunoglobulin formation in serum and saliva. Furthermore, the carrier rate of pathogenic bacteria in their throats was investigated, and in some of the children, who had their adenoids removed, immunofluorescent studies were performed. The study revealed highly reduced levels of serum and saliva IgA in the *younger* children with asthma. In addition, in these children a connection between recurring respiratory infections and high carrier rate of presumably pathogenic bacteria was observed. Also in the *older* children, significantly reduced levels of serum and saliva IgA compared with age related controls were *found*, but these children did not have an increased frequency of pathogenic bacteria or respiratory infections. In addition, low levels of serum IgM were found in the older children with asthma. *The results* of the study support the theory that low IgA levels facilitate the entrance of pathogenic bacteria through the epithelial surfaces, resulting in an overstimulation of the IgE system and the development of bronchial asthma in the younger children. In the younger as well as in the older patients, a high frequency of atopy among the closest relatives was observed.

Key words: IgA levels; bacterial carrier rate; bronchial asthma.

P. Aabel Østergaard, Department of Pediatrics, Aalborg Hospital North, DK-9000 Aalborg, Denmark.

Received 15 . 76   Accepted 17.1.77

In recent years, immaturity of the IgA system in infants and young children of atopic parents is postulated to lead to abnormal entry of antigens through mucosal surfaces, secondarily leading to an overstimulation of the IgE system and the development of atopic diseases (*Taylor et al.* 1973).

The aim of the present study was to investigate the immunoglobulin formation, the carrier rate of pathogenic bacteria, and the frequency of respiratory infections in children with confirmed asthmatic symptoms in order to test the theory that low IgA levels and carriage of pathogenic bacteria in the throat are connected with the development of bronchial asthma.

MATERIALS AND METHODS

The patients. The series comprises 54 children, who were admitted to the Department of Pediatrics, Aalborg Hospital, North, because of bronchial

asthma in the period June 1974 to July 1975. Originally they constituted a group of 63 children, but only 54 of them, who were reexamined during the period May to June 1976 fulfilled the following diagnostic criteria: (1) more than 3 years of age, (2) duration of symptoms for more than 2 years, (3) a typical history of bronchial asthma often with seasonal variations, and (4) relief of symptoms at hospitalization by the administration of bronchodilatators and/or corticosteroids.

During hospitalization all the children and their parents were interviewed by a medical doctor with regard to common health, the frequency of respiratory infections, seasonal variation of asthmatic symptoms and possible allergens provoking their disease and the frequency of atopic diseases among their closests relatives. Prick test on the skin of the forearm were performed in most of the children with commercial allergens, purchased from Allergologisk Laboratorium, Copenhagen on admission and were repeated as often as indicated during their hospitalization. A radioallergosorbent (RAST) test performed by Medicinsk Laboratorium Copenhagen, was done on the children with a positive (more than + + +) response to the prick test with one or more of the provided allergens.

Before the reexamination the children were divided into 2 groups: (1) patients with a typical history of recurring respiratory infections, which often preceded their asthmatic symptoms. (32 children, referred to as *Group I*). (2) Patients without frequently occuring respiratory infections (22 children, referred to as *Group II*). At the reexamination, the patients and the parents were interviewed by the author with regard to the frequency of asthmatic symptoms. On that occasion, 6 children were excluded either because they were very young (4) or no longer had asthmatic symptoms. In the intervening time 3 children had moved out of the area and were therefore no longer avaliable for study. The remaining 54 children had a medical check up, serum and saliva were collected for immunoglobulin determination, and a swab was taken from the throat for bacterial culture.

Controls. 54 healthy controls from the same geographical area as the patients were selected with regard to age and sex to match the patients. None of the controls had experienced severe respiratory infections or atopic diseases, and none of the controls had close relatives with allergic diseases. Furthermore they had not had an earlier adenoidectomy or tonsillectomy. The throat was inspected in all the controls to make sure that the tonsils were in situ. *Serum and saliva samples* were collected from all the patients and the controls and were frozen immediately at − 20° C. The saliva was always tested for blood contamination with haemastix and found negative. None of the patients or the controls offered signs of inflammatory processes of the mouth.

Serum and saliva IgG, IgA and IgM assays were performed by the Department of Clinical Chemistry, Aalborg Hospital South. Rabbit-anti-human-IgG, IgA and IgM obtained from Dacopatts, Copenhagen, were used. Details concerning the methods used are presented elsewhere (*Østergaard & Blom Østergaard* 1976). Pooled human serum, calibrated in terms of the WHO reference standard preparation (*Rowe et al.* 1972) was used as standard in serum as well as in saliva immunoglobulin estimations. The sensitivity for detection of saliva immunoglobulins was 0.09, 0.54 and 2.04 I.U/ml for IgG, IgA and IgM respectively. A control serum was included in every run. The day to day precision of the runs (n = 10 days) was 6.8 per cent, 5.4 per cent and 5.9 per cent for IgG, IgA and IgM respectively.

Serum IgE estimations were performed by Medicinsk Laboratorium, Copenhagen using a radioimmunosorbent test (Phadebas kit, Pharmacia, Copenhagen). The sensitivity for detection of IgE in serum was 10 U/ml.

Bacterial cultures were performed by the Department of Medical Microbiology, Aalborg Hospital, South. Within half an hour of the throat swabs being taking, they were spread onto blood agar and chocolate agar plates. Incubation was at 35° C aerobically and anaerobically and the plates were read after 24 and 48 hours. Details concerning the characteristics used for identification of the various pathogens are published elsewhere (*Østergaard* 1976). *Immunofluorescent studies* were performed on adenoids excised from 11 of the patients included in Group I. The adenoidectomy was performed on the children during the period October 1975 and May 1976 mainly because of chronic otitis media and recurring bronchitis. Details concerning tissue preparation, the technique and the light and fluorescense microscope used have been published earlier (*Østergaard* 1975). The rabbit-anti-human-IgG, IgA, IgM and IgE sera used were purchased from Behringwerke AG, Germany. The batch numbers and their Fluorescein/Protein (F/P) molar ratios were as follows: IgG, batch number 687 D (F/P molar ratio 2.6), IgA, batch number 678 E (F/P molar ratio 2.6), IgM, batch number 693 E (F/P molar ratio 2.9), IgE, batch number 685 C (F/P molar ratio 3.1). Blocking tests were carried out by exposing the sections to (1) unlabelled rabbit anti human IgG, IgA, IgM and IgE and (2) unlabelled rabbit-antihuman-albumin. The details are published elsewhere (*Østergaard* 1975). The results of the immunofluorescence studies are expressed as − (no fluorescing cells), + (−) (occasionally a single cell), + (2–4 cells), + + (5–10 cells), + + + (10–20 cells) and + + + + (more than 20 cells) in sections of dimensions of approximately 5 × 5 mm.

Statistical evaluation of the results was performed by the Mann-Whitney U test for unpaired

TABLE 1 *Relationship between Bacterial Culture, other Atopic Disease than Asthma, and the Frequency of Atopic Diseases in the closest Relatives among the Patients with Recurrent Respiratory Infections*

Patient number	Age (years)	Bacterial culture at admission	Bacterial culture at reexamination	Other atopic diseases	Atopic diseases in closest relatives
1	4 2/12	*H influenzae*	Normal flora	—	+
2	5 2/12		Normal flora	—	—
3	4 7/12	Normal flora	Normal flora	+	—
4	2 6/1	Meningococci	Pneumococci	—	+
5	4	Pneumococci	Pneumococci	—	+
6	7 6/12	Normal flora	Normal flora	+	+
7	4 2/12	*H influenzae*	*H influenzae*	—	+
8	5	Pneumococci	Pneumococci	+	+
9	12 6/12	Pneumococci	Normal flora	+	—
10	7 1/12	*H influenzae*	Normal flora	—	+
11	7	*H influenzae*	Normal flora	—	—
12	3 1/12	Normal flora	Pneumococci	—	+
13	4 3/12	Pneumococci	Normal flora	+	+
14	7 10/12	Normal flora	Normal flora	—	
15	5 2/12	*H influenzae*	*H influenzae*	—	+
16	7 2/12	Pneumococci	Normal flora	—	—
17	3 3/12	Normal flora	Normal flora	—	—
18	10 7/12	Pneumococci	Normal flora	+	+
19	5 10/12	*H influenzae*	Pneumococci	—	+
20	6 8/12	Normal flora	Normal flora	—	+
21	5 3/12	*H influenzae*	Normal flora	—	—
22	5 5/12	*H influenzae*	Pneumococci	—	+
23	3 1/12	Pneumococci	Normal flora	—	+
24	3 1/12	*H influenzae*	Normal flora	—	+
25	8 4 12	Pneumococci	Pneumococci	+	+
26	4 4/12	*H influenzae*	Normal flora	—	+
27	8 1/12	*H influenzae*	Normal flora	+	+
28	5 5/12	Normal flora	Pneumococci	+	+
29	4 8/12	*H influenzae*	Pneumococci	—	+
30	6 6/12	Normal flora	*H influenzae*	+	+
31	3 11/12	Pneumococci	Normal flora	—	+
32	4 10/12	Pneumococci	Normal flora	—	+

observations. The confidence limits of the observations were chosen at the 5 per cent level of significance. P values ≤ 0.05 were considered significant.

RESULTS

Examination of the patients on admission and at the reexamination. The patients included in Group I are listed in Table 1. The mean age of the patients was 5 / years, and the mean age of their controls was 5 / years. It appears from the table that a majority of these patients harboured presumably pathogenic bacteria during their stay at hospital (74 per cent) but it should be stressed that most of these patients were admitted to the hospital because of acute respiratory infections in connection with symptoms of bronchial asthma. On admission, the overall dominating pathogens were pneumococci and *Haemophilus influenzae*. At the reexamination of the patients, all, except one, offered no signs of inflammatory processes. Regardless of this, 9 of the patients harboured pneumococci and 3 harboured *Haemophilus influenzae* (37.5 per cent) in their throats. In this group of

TABLE 2 *Relationship between Bacterial Culture other Atopic Diseases than Asthma and the Frequency of Atopic Diseases among the closest Relatives in the Patients without Recurrent Respiratory Infections*

Patient number	Age (years)	Bacterial culture at admission	Bacterial culture at reexamination	Other atopic diseases	Atopic disease in closest relatives
1	12 4/12	Normal flora	Normal flora	+	+
2	10	Normal flora	Normal flora	—	+
3	12 3/12	Normal flora	Normal flora	+	+
4	7 11/12	Normal flora	Normal flora	+	+
5	9 5/12	Normal flora	Normal flora	—	—
6	11 1/12	beta haemolytic streptococci	Normal flora	+	+
7	11 1/12	Normal flora	Normal flora	+	—
8	15 8/12		Normal flora	—	+
9	10	Normal flora	Normal flora	+	+
10	10	Normal flora	Normal flora	+	—
11	8 6/12	Pneumococci	Normal flora	—	—
12	11 6/12	Normal flora	Pneumococci	+	+
13	15 1/12		Normal flora	+	+
14	13 3/12	Normal flora	Pneumococci	+	—
15	14 6/12	Normal flora	Normal flora	—	+
16	7 3/12	Pneumococci	Normal flora	+	+
17	15 5/12	Normal flora	Pneumococci	—	+
18	10 5/12	Pneumococci	Normal flora	+	+
19	9 6/12	Normal flora	Normal flora	+	+
20	13 3/12	Normal flora	Normal flora	—	+
21	9 5/12		Normal flora	—	—
22	12 2/12	Normal flora	Normal flora	+	+

patients, 31 per cent had other atopic manifestations than asthma, mainly atopic dermatitis and allergic rhinitis. Among the closest relatives (mothers, fathers, sisters and brothers) of Group I patients, atopic diseases in 77 per cent were found

The patients in *Group II* are listed in Table 2 The mean age of these patients was 11 $^1/_2$ years, and the mean age of their controls was 11 $^2/_{12}$ years. During admission none of these patients offered signs of infections, but in 4 of 19 (21 per cent) pathogenic bacteria were cultured from their throats. At the reexamination 3 patients harboured pneumococci (9 per cent) In this group 64 per cent of the patients had other atopic diseases than asthma Among the closest relatives of the patients in Group II a frequency of atopic diseases of 77 per cent was found which was equivalent to the 77 per cent observed among relatives to the patients in Group I

Sixty one per cent of the patients in Group II had a adenoidectomy or a tonsillectomy performed in their early childhood because of recurrent respiratory infections.

Prick tests were performed in 24 Group I patients, and 21 of them were positive to one or more allergens (average 2 allergens) All the patients in Group II had prick tests performed and showed positive reactions to a great number of allergens (average 9 allergens) In 64 per cent Group I patients and in 72 per cent Group II patients a positive prick test to a given allergen corresponded with a positive RAST test to the same allergen

Serum immunoglobulin levels Ranges (including 95 per cent ranges) and means (medians) of the results of the serum immunoglobulin estimations in the patients and the controls are listed in Tables 3 and 4 In patients *Group I* (Table 3) a very low mean

TABLE 3 *Range and Means (Medians) of Serum and Saliva Immunoglobulins in the Patients with recurrent Infections and their Controls*

	Serum Immunoglobulins IU/ml				Saliva IgA IU/ml
	IgG	IgA	IgM	IgE	
Patients					
Range	18–175 (54–174)	0–78 (5–69)	18–182 (41–178)	18–1035 (18–970)	0–4.2 (0.6–4.0)
Mean	98	27	96	200	1.8
Controls					
Range	52–178 (64–172)	32–164 (44–161)	16–218 (32–202)	15–270 (18–268)	2.2–8.4 (2.4–7.6)
Mean	94	85	85	58	5.8
P =	>0.2	<0.001	>0.2	<0.01	<0.001

93 per cent ranges in parentheses

IgA level of 27 IU/ml was found, which, compared with a mean IgA level of 85 IU/ml in the controls, was significant ($p < 0.001$). With regard to serum IgG and IgM in the patients, the mean levels (94 and 85 IU/ml) did not differ from the mean IgG and IgM levels (98 and 96 IU/ml) in the controls ($p > 0.2$). In patients *Group II* (Table 4) a mean serum IgA of 67 IU/ml in the patients was slightly but significantly reduced compared with a mean serum IgA of 85 IU/ml in the controls ($p < 0.05$). With regard to serum IgG a mean of 95 IU/ml was compared with a mean IgG of 106 IU/ml in the controls ($p > 0.1$) while mean serum IgM (67 IU/ml) was rather low in this group of patients compared with a mean IgM of 124 IU/ml in their controls ($p < 0.01$).

Serum IgE levels of the patients and their controls are also given i Tables 3 and 4. In patients *Group I* (Table 3) a mean IgE of 200 IU/ml gave a significant difference com-

TABLE 4 *Range and Means (Medians) of Serum and Saliva Immunoglobulins in the Patients without Recurrent Infections and their Controls*

	Serum Immunoglobulins IU/ml				Saliva IgA IU/ml
	IgG	IgA	IgM	IgE	
Patients					
Range	60–150* (62 150)	18–126 (24–126)	14 140 (18–140)	100–4000 (140–4000)	0.8–12.5 (0.8–6.2)
Mean	95	67	67	725	3.1
Controls					
Range	52 158 (61 158)	28–176 (42 176)	22 242 (48–242)	20–500 (24–500)	2.2–11.3 (2.3–9.4)
Mean	106	85	124	63	5.5
P	>0.1	<0.05	<0.01	<0.01	<0.01

93 per cent ranges in parentheses

TABLE 5 *Correlation between the Immunofluorescent Studies on Adenoids and Serum and Saliva Ig levels in the Patients with Recurrent Respiratory Infections*

Patient Number	Immunofluorescent Studies IgG	IgA	IgM	IgE	Serum Immunoglobulins U/ml IgG	IgA	IgM	IgE	Saliva IgA U/ml
4	+ + +	+ (—)	+	+ +	93	13	67	701	2.2
5	+ +	—	+ +	+ +	101	32	123	550	2.2
7	+ +	—	+ +	+ +	67	11	77	440	2.1
8	+ +	—	+	+	75	28	68	34	0.6
11	+	—	+	+ +	42	5	29	880	1.1
17	+ + + +	+ +	+ +	+ +	124	42	246	110	2.4
22	+ + +	+	+	+	84	14	86	380	1.1
23	+ + +	+	+ +	+	110	33	140	220	2.1
25	+ +	+	+	—	60	69	67	88	2.4
28	+ + +	—	+ +	+ +	170	0	67	630	0
32	+ + +	+ (—)	+	+	102	24	118	330	1.1
Mean (Median)					93	24	77	330	2.1

pared with a mean serum IgE of 58 IU/ml in the controls ($p < 0.01$). In patients *Group II* (Table 4) a mean serum IgE of 725 IU/ml was also very different from a mean serum IgE of 63 IU/ml in their controls ($p < 0.01$).

Saliva IgA levels of the patients and the controls are listed in Tables 3 and 4. In patients *Group I* (Table 3) a mean saliva IgA of 1.8 IU/ml was different from a mean saliva IgA of 3.8 IU/ml in the controls ($p < 0.001$). In patients *Group II* (Table 4) a mean saliva IgA of 3.1 IU/ml was also different from a mean saliva IgA of 5.5 IU/ml in the controls ($p < 0.01$). With regard to saliva IgG this immunoglobulin was only found in 10 of the 54 patients and saliva IgM was only found in 4 of the patients. Therefore, a comparison between patients and controls with regard to those immunoglobulins could not be made.

Immunofluorescense studies. The results of the immunofluorescent studies and the serum and saliva Ig levels of the 11 patients in *Group I* are given in Table 5. IgG fluorescing plasma cells were found in great numbers in all the adenoids investigated and also IgM fluorescing plasma cells were invariably found. Conversely IgA fluorescing plasma cells lacked in 5 of the patients and were scarcely represented in another 2. With regard to IgE fluorescing plasma cells, these were found in great numbers and were only lacking in 1 patient. The results of the serum and saliva Ig levels obtained in the patients undergoing adenoidectomy only confirmed the results obtained in all the patients in Group I.

DISCUSSION

The very low serum and saliva IgA levels in the younger patients in this study seemed to be connected with a rather high carrier rate of presumably pathogenic bacteria. Furthermore, the older patients with asthma had slightly reduced serum IgA levels and more distinctly reduced saliva IgA levels compared with agerelated controls. In this group of patients, the carrier rate of pathogenic bacteria was only 9 per cent which is comparable with the bacterial carrier rate among healthy school children (*Cornfield* 1961, *Turk* 1963). In addition the older patients had rather low serum IgM levels.

For some years already several studies had indicated a connection between immaturity of the IgA system and the development of atopic diseases in children. Hence *Kaufman* & *Hobbs* (1970) found an incidence of IgA deficiency of 7 per cent in a large population

of atopic individuals. Furthermore, *Waldman et al.* (1970) reported on a defect in secretory antibody production, but not in serum antibody synthesis, to influenzae aerosol immunization in 5 of 8 patients with bronchial asthma and severe, recurrent respiratory infections. *Taylor et al.* (1973) in infants of atopic parents, found a significantly reduced maturation on the IgA system in connection with the development of atopic diseases and postulated that the immature IgA system observed in these infants was responsible for atopy. *Stokes et al.* (1974) who investigated serum IgA and IgE antibodies to the house dust-mite *Dermathophagoides farinae* in skin-test negative and skin-test positive adults, found significantly more IgE antibody and significantly less IgA antibody to this allergen in the skin-test positive individuals. The authors postulated that adult atopics, in spite of normal or even elevated serum IgA levels, had a qualitative abnormality of the IgA antibody response.

Conversely *Buckley et al.* (1968) in 85 allergic children without complicating illness, found no significant reduction in serum IgA. In addition, *Salvaggio et al.* (1973) in studies on nasal and sputum IgA in 21 atopic and 19 nonatopic subjects, found no difference in IgA between these two groups. They postulated that raised mucosal absorption of inhalant allergens by atopics could not be accounted for on the basis of a local protective exocrine IgA deficiency per se.

The diverging results of these studies are difficult to explain, but may be the result of the selection of the patients. In the present study the dividing of the patients into 2 groups, one with and the other without recurrent respiratory infections preceding their asthmatic symptoms, seems to be a practicable model in studies on the influence of IgA deficiency and bacterial carrier rate on the development of bronchial asthma in children. On the basis on this division, a considerable connection between low levels of IgA in serum and secretions on the one hand and a high carrier rate of pathogenic bacteria on the other seemed to exist in the younger patients. In addition, the older patients had a high frequency of adenotonsillectomy in their early childhood, which indicates that the children at that time had an increased tendency to develop respiratory infections. Furthermore, these children had low saliva IgA levels, and although a considerable influence of saliva flow rate must be accounted for (*Hansson & Brandtzaeg* 1973) the low levels obtained is in favour of the theory that a delayed maturation of the secretory IgA system facilitates the development of bronchial asthma.

The low serum IgM levels observed in the older patients in this study may be due to another immunological defect, and low IgM levels in asthmatics have also been found by others (*Kaufman & Hobbs* 1970). Often a "compensatory" increase of IgM is observed in individuals with IgA deficiency (*Stocker et al.* 1968, *Collins-Williams et al.* 1972, *Savilahti* 1973) but *Goldberg et al.* (1968) did not find such a compensatory IgM increase in IgA deficient subjects. The ratio of IgG to IgA in bronchoalveolar fluid has been found to be 2.5:1 whereas in nasal secretions it was 1:3 (*Waldman et al.* 1973). Furthermore, following intraveneous and intrapulmonary immunization in dogs with sheep erythrocytes, an increase in bronchoalveolar spaces with IgG and IgM-antibody secreting cells was observed, while IgA-secreting cells were not detected (*Kaltreider et al.* 1974). These studies suggest a subdivision within the respiratory compartment i.e., predominantly secretory IgA antibody formation of the upper respiratory tract and IgG and perhaps IgM antibody formation of the lower respiratory tract. According to this view the low IgM levels observed in some patients with bronchial asthma may reflect an insufficiently operating immunologic defence mechanism of the lower respiratory tract.

The conclusions, which can be drawn from this study are in agreement with the observations that a defective development of the IgA system favours the adherence and the colonization of pathogens on the mucosal surfaces (*Gibbon & Van Houte* 1971). This may cause repeated damage of the epithelium du-

TABLE 5 *Correlation between the Immunofluorescent Studies on Adenoids and Serum and Saliva Ig levels in the Patients with Recurrent Respiratory Infections*

Patient Number	Immunofluorescent Studies IgG	IgA	IgM	IgE	Serum Immunoglobulins U/ml IgG	IgA	IgM	IgE	Saliva IgA U/ml
4	+ + +	+ (—)	+	+ +	93	13	67	201	22
5	+ +	—	+ +	+ +	101	32	123	550	22
7	+ +	—	+ +	+ +	67	11	77	440	21
8	+ +	—	+	+	75	[illegible]8	68	34	06
11	+	—	+	+ +	42	5	29	880	11
17	+ + + +	+ +	+ +	+ +	124	42	246	110	24
22	+ + +	+	+	+	84	14	86	380	11
23	+ + +	+	+ +	+	110	33	140	2[illegible]0	21
25	+ +	+	+	—	60	69	67	88	24
28	+ + +	—	+ +	+ +	170	0	67	630	0
32	+ + +	+ (—)	+	+	102	24	118	330	11
Mean (Median)					93	24	77	330	21

pared with a mean serum IgE of 58 IU/ml in the controls ($p < 0.01$). In patients *Group II* (Table 4) a mean serum IgE of 725 IU/ml was also very different from a mean serum IgE of 63 IU/ml in their controls ($p < 0.01$).

Saliva IgA levels of the patients and the controls are listed in Tables 3 and 4. In patients *Group I* (Table 3) a mean saliva IgA of 1.8 IU/ml was different from a mean saliva IgA of 3.8 IU/ml in the controls ($p < 0.001$). In patients *Group II* (Table 4) a mean saliva IgA of 3.1 IU/ml was also different from a mean saliva IgA of 5.5 IU/ml in the controls ($p < 0.01$). With regard to saliva IgG this immunoglobulin was only found in 10 of the 54 patients, and saliva IgM was only found in 4 of the patients. Therefore a comparison between patients and controls with regard to those immunoglobulins could not be made.

Immunofluorescense studies. The results of the immunofluorescent studies and the serum and saliva Ig levels of the 11 patients in *Group I* are given in Table 5. IgG fluorescing plasma cells were found in great numbers in all the adenoids investigated and also IgM fluorescing plasma cells were invariably found. Conversely IgA fluorescing plasma cells lacked in 5 of the patients and were scarcely represented in another 2. With regard to IgE fluorescing plasma cells, they were found in great numbers and were only lacking in 1 patient. The results of the serum and saliva Ig levels obtained in the patients undergoing adenoidectomy only confirmed the results obtained in all the patients in Group I.

DISCUSSION

The very low serum and saliva IgA levels in the younger patients in this study seemed to be connected with a rather high carrier rate of presumably pathogenic bacteria. Furthermore, the older patients with asthma had slightly reduced serum IgA levels and more distinctly reduced saliva IgA levels compared with agerelated controls. In this group of patients, the carrier rate of pathogenic bacteria was only 9 per cent which is comparable with the bacterial carrier rate among healthy school children (*Cornfield* 1961, *Turk* 1963). In addition, the older patients had rather low serum IgM levels.

For some years already several studies had indicated a connection between immaturity of the IgA system and the development of atopic diseases in children. Hence *Kaufman & Hobbs* (1970) found an incidence of IgA deficiency of 7 per cent in a large population

deficiencies in an topic population. Lancet ii 1061 1063 1970.

Polmar S H., Waldmann, T A. & Terry W D The relationship of IgA and IgE deficiency Birth Defects *II* 147–151 1975.

Polmar S H Waldmann, T A & Balestra, S T Immunoglobulin E in immunologic deficiency diseases I Relation of IgE and IgA to respiratory tract disease in isolated IgE deficiency IgA deficiency and ataxia telangiectasia. J Clin Invest. *51* 326–330, 1972.

Luckasen J R Sabad A Goltz R W & Kersey J H T and B-lymphocytes in atopic eksema Arch. Derm. *110* 375–377 1974

Rowe D S A derson S G & Grab B A research standard for human serum immunoglobulins IgG IgA and IgM. Bull. Wld. Hlth. Org. *42* 535–538, 1972.

Salvaggio J Lopez M Arquembourg P., Waldman R & Sly M Salivary nasal wash, and sputum IgA concentrations in atopic and nonatopic individuals, J Allerg Clin. Immunol *51* 335–343 1973

Savilahti E IgA deficiency in children, Immunoglobulin-containing cells in the intestinal mucosa, immunoglobulins in secretions and serum IgA levels Clin. exp. Immunol. *13* 395–406, 1973

S hwartz, D P & B ckley R H Serum IgE concentrations and skin reactivity to anti-IgE antibody in IgA deficient patients New Engl. J Med *284* 513–517 1971

Stocker F Ammann P & Rossi E Selective gamma A-globulin deficiency with dominant autosomal inheritance in a Swiss family Arch. Dis Child. *43* 585–593, 1968.

Stokes C R Taylor B. & Turner M W Association of housedust and grass-pollen allergies with specific IgA antibody deficiency Lancet ii 485–488, 1974

Tada T., Okumura K. & Taniguchi, M.: Regulation of homocytotropic antibody formation in the rat. VII. Carrier functions in the anti-hapten homocytotropic antibody response. J Immunol *108* 1535–1540, 1972.

Taylor B., Norman A Orgel H., Stokes, C., Turner M & Soothill, J F.: Transient IgA deficiency and pathogenesis of infantile atopy Lancet ii 11–114 1973

Turk, A., Lichtenstein L. M & Norman P S. Nasal secretory antibody to inhalant allergens in allergic and non-allergic patients. Immunology *19* 85–95 1970.

T rk, D C Naso-pharyngeal carriage of Haemophilus type b J Hyg. Camb. *61* 247–256 1963

Waldman R H Jurgensen P F & Olsen G N. The immune response of the human respiratory tract I Immunoglobulin levels and influenza vaccine antibody response J Immunol. *111* 38-41 1973.

Waldman R. H., Newman M & Greenspan R. Immunologic response of young patients with asthma and/or recurrent respiratory infections to aerosol influenza immunization. J Asth. Res. *7* 191–197 1970

Østergaard P A Presence of immunoglobulin-containing plasma in tonsils from tonsillectomized children. Acta path. microbiol. scand. Sect. C, *83* 406-412 1975

Østergaard P Aa. IgA levels and carrier rate of *Haemophilus influenzae* and beta-haemolytic streptococci in children undergoing tonsillectomy Acta path. microbiol. scand. Sect. C, *84* 290–298 1976.

Østergaard P Aa. IgA levels and carrier rate of pathogenic bacteria in 27 ch ldren previously tonsillectomized. Acta path. microbiol. scand. Sect. C, *85* 178–186, 1977

Østergaard P Aa. & Blom M (unpublished results)

ring acute infection and next, facilitate the entrance of the pathogens through the mucosa, subsequently stimulating the IgE system and the development of atopy. The blocking activity to ragweed antigen and to rye grass antigen in nasal secretions of humans has been found to reside in IgA (*Turk et al* 1970) and *Polmar et al* (1972) hypothesized that in the absence of IgA antibodies, IgE antibodies synthesized locally might interact with bacterial viral or other antigens, leading to the release of histamin and the creation of mucosal congestion and transudation of fluids which favour the growth of pathogenic organisms in the respiratory tract tissues. Furthermore, *Schwartz* & *Buckley* (1971) found a prevalence of atopy of 73 per cent in 26 children with IgA deficiency. These findings suggest that secretory immune system may play a role in limiting the antigenic stimulation of the IgE system.

However this theory is not in agreement with the observations that apparently healthy individuals can lack IgA in serum and secretions (*Johansson et al* 1968 *Polmar et al* 1975) and other factors may be responsible for triggering the IgE system. It seems reasonable to suggest that a hereditary factor may be partly responsible, as a high frequency of atopy among the closest relatives of the patients was observed in this study. Furthermore in a study of the patients with chronic tonsillitis (*Østergaard* 1975 *Østergaard* 1976) a $2^1/_2$ year follow up of some of the patients (*Østergaard* 1977) revealed that in the intervening time 3 patients with low IgA had developed atopic disease and in these 3 cases allergic diseases were also found among the closest relatives.

The possible interaction between the IgA and IgE systems may be initiated via subpopulations of T-cells with helper or suppressor functions on the IgA and IgE antibody production (*Clough et al* 1971 *Tada et al* 1972). The recent observations of a reduced T-cell function in patients with atopic dermatitis (*Luckasen et al* 1974 *Andersen* & *Hjorth* 1975) is in favour of the theory that the immature development of the IgA antibody system and the increased activity of the IgE-system observed in the present patients may be the result of a defective cooperative interaction with T lymphocytes.

The studies were supported by *Det Lægevidenskabelige Forskningsfond for Nordjyllands Amt*. The author is indepted to Dr *K E. Sørensen*, Aalborg, for selection of healthy controls.

REFERENCES

Andersen E. & *Hjorth, N* B-lymphocytes, T lymphocytes and phytohemagglutinin responsiveness in atopic dermatitis. Acta Dermato-Venerol. 55 345–349 1975

Buckley R H Dees S C & *O'Fallon W M* Serum immunoglobulins II Levels in children subject to recurrent infections. Pediatrics 41 50–60 1968

Clough J D., Mims L. H & *Strober W* Deficient IgA antibody response to arsanilic acid bovine serum albumin (BSA) in neonatally thymectomized rabbits. J Immunol. 106 1624–1629 1971

Collins Williams C Kokubu H C., Lamenza, C Nizami R., Chiu A W Lewis McKinley C., Comerford T A & *Varga E A.* Incidence of isolated deficiency of IgA in the serum of Canadian children. Ann Allerg. 30 11–23 1972

Cornfield D A four-year study of the occurrence of beta-haemolytic streptococci in 64 school children. New Engl. J Med. 264 211–215, 1961

Gibbons R J & *Van Houte J* Selective bacterial adheration to oral epithelial surfaces and its role as an ecological determinant. Infect Immun. 3 567–572, 1971

Goldberg, L. S Barnett E. & *Fudenberg, H H* Selective absence of IgA A family study J Lab Clin. Med 72 204–212 1968

Hansson L. A Brandtzæg P Immunologic disorders in infants and children. In *Stiehm E. R.* & *Fulginiti V A* (Eds.) Secretory antibody system. W B Saunders Company Philadelphia, 1973

Johansson S G O Högman E. F & *Killander J* Quantitative immunoglobulin determinations. Acta path. microbiol scand. 74 519–524 1968

Kaltreider H B Kyselka L. & *Salmon S E* Immunology of the lower respiratory tract. 2. The plaque-forming response of canine lymphoid tissues to sheep erythrocytes after intrapulmonary or intravenous immunization. J Clin. Invest. 54 263–270 1974

Kaufman H S & *Hobbs J R.* Immunoglobulin

1 Preparation of the Antigen( )

A femoral artery without macroscopical atherosclerosis from man who had been dead for about twelv hours was used for the production of arterial antigen( ). After skin location on the medial side of the thigh the femoral artery was removed under optimal aseptic conditions. Fragments of the artery including tunica intima, tunica media and tunica adventitia, were subsequently homogenized immediately by homogenizer pestles. The homogenization time was five minutes at 4 C. Finally the homogenate was extracted in Hanks' buffered salt solution overnight at 4 C. After centrifugation the supernatant was pipetted off from the sediment and the concentration of the protein in the supernatant was measured.

2 Leucocyte Migration Technique

The migration of the leucocytes from both patients suffering from borderline essential hypertension and normal persons was studied in culture chambers containing an arterial protein concentration of about 50 micrograms per ml. This concentration of arterial protein in the culture chambers has been shown by titration experiments to be optimal (*Olsen & Lefi* 1973; *Olsen & Lefi* unpublished results). The results were calculated as the migration index, which is defined as the ratio between the migration area of antigen-containing and antigen-free cultures.

An evaluation of the specificity of the leucocyte migration technique in the experimental situation pertaining to hypertensive patients has been made previously and it has been shown that the method is specific (Olsen & Lefi 1973). In the present study the specificity of the method was examined in 5 of the 19 hypertensive patients. In these cases the migration in culture chambers containing femoral arterial proteins was compared with the migration in culture chambers containing equal concentrations of protein from homogenized and extracted peritoneal and liver tissue derived from human subjects.

The precision of the method expressed as the coefficient of variation was 5 per cent.

3 Patient Material

This consisted of patients suffering from borderline essential hypertension. A uniformly accepted definition of borderline hypertension is not available but this could best be described as a condition of elevated blood pressure with readings not sufficiently high to warrant early treatment (*Julius & Esler* 1975). Another way to define borderline hypertension is those patients who sometimes are hypertensive and at other times appear to be normotensive, the normal blood pressure being 140/90 mm Hg (*Julius et al.* 1974).

The present patient material consisted of 10 women and 9 men. The mean age of the patients was 57 years and the mean blood pressure was 150/100 mm Hg. Only two patients were treated with antihypertensive drugs at the time of the study. The patients had been referred to hospital by the general practitioner because of occasionally elevated blood pressure. None of the patients showed any hypertensive changes as judged by X-ray of the heart, electrocardiography and examination of the background of the eye. The renal function was normal. There were no grounds for assuming that the borderline hypertension was secondary hypertension.

4 Control Persons

19 healthy persons of the same sex and age as the patient group, but without arterial hypertension or other diseases, were used as controls.

RESULTS

The results obtained by the leucocyte migration technique are shown in Fig. 1. A statistical calculation shows that the migration indices from the borderline hypertensive patients are significantly different from the normotensive controls, $P<0.005$ (Wilcoxon Mann Whitney rank sum test). This shows that the borderline hypertensive patients have an inherent hypersensitivity of the delayed type directed against arterial wall components.

The specificity of the migration technique was examined in culture chambers which contained extracted protein from peritoneal and liver tissue in a concentration equal to the arterial protein concentration. Results from five such experiments on material from borderline hypertensive patients showed a migration practically identical to that in control chambers, thus showing that the method was specific.

DISCUSSION

These results show that some patients suffering from borderline essential hypertension seem to have a delayed type of hypersensitivity directed against arterial wall components. These results are in agreement with previously published results which demon-

Acta path microbiol scand Sect C 85 196–198, 1977

DELAYED HYPERSENSITIVITY AND BORDERLINE ESSENTIAL HYPERTENSION

Delayed Hypersensitivity Against Arterial Wall Components in Patients suffering from Borderline Essential Hypertension

FINN OLSEN and STEN RASMUSSEN

Institute of Hygiene, University of Copenhagen, and
Medical Department C, Diakonissestiftelsen, Copenhagen Denmark

Olsen, F & Rasmussen S Delayed hypersensitivity and borderline essential hypertension. Delayed hypersensitivity against arterial wall components in patients suffering from borderline essential hypertension. Acta path. microbiol. scand Sect. C 85 196–198 1977

By means of the leucocyte migration technique, it has been demonstrated that the migration indices from 19 patients suffering from borderline essential hypertension are significantly different from those of 19 normal persons. These results support the view that delayed hypersensitivity directed against arterial wall components is a possible pathogenetic factor in patients suffering from essential hypertension.

Key words Delayed hypertension borderline essential hypertension leucocyte migration.

F Olsen, Institute of Hygiene, University of Copenhagen, 21 Blegdamsvej DK 2100 Copenhagen Ø Denmark.

Received 22.xi 76 Accepted 21.xii.76

Hypersensitivity of the delayed type directed against arterial wall antigens seems to be involved in the hypertensive vascular disease in animals with experimental arterial hypertension (*Olsen* 1971 *Gerner Svendsen* 1975 1976) The thymus of spontaneously hypertensive rats shows morphological changes which suggest the involvement of an autoimmunological mechanism in the development of the vascular lesions (*Rojo-Ortega et al* 1973) Furthermore it is possible to demonstrate in patients suffering from *severe* essential arterial hypertension hypersensitivity of the delayed type against arterial wall antigens, thus indicating that delayed hypersensitivity is a pathogenetic factor in at least some cases of essential hypertension (*Olsen* 1974)

The aim of the present study has been to examine whether it is possible to demonstrate delayed hypersensitivity directed against arterial wall components in patients suffering from essential *borderline* hypertension. If this were so, the results would support the hypothesis that a delayed type of hypersensitivity could be a pathogenetic factor in some cases of essential hypertension.

MATERIALS AND METHODS

The leucocyte migration technique was used for in vitro examination of the delayed type of hypersensitivity (*Søborg & Bendixen* 1967 *Bendixen & Søborg* 1969)

Acta path. microbiol. scand. Sect. C, 85 199–206, 1977

CELL-MEDIATED IMMUNE RESPONSE TO GUINEA PIG AND BOVINE BASIC PROTEINS OF MYELIN IN LEWIS AND PVG RATS AND THEIR HYBRIDS

JOHANNES LINDH

Tornblad Institute, University of Lund, Lund, Sweden

Lindh, J. Cell-mediated immune response to guinea pig and bovine basic proteins of myelin in Lewis and PVG rats and their hybrids. Acta path. microbiol. scand. Sect. C, 85 199–206 1977

The *in vitro* cell-mediated immune response of rat lymph node cells (LNC) to guinea pig and bovine encephalitogenic protein (EP) has been studied with LNC transformation test. LNC were obtained from either Lewis, PVG or F (Lewis x PVG) rats 28 days after challenge with guinea pig or bovine EP in Freund' complete adjuvant (FCA) of different mycobacterium content. N differences between the strains or their hybrids in LNC response could be shown if stimulated with sensitizing EP despite the great differences in capacity for disease development in those animals. Nor did fivefold lowering of the amount of mycobacterium content in FCA, when guinea pig EP was used at challenge, lessen the in vitro response, although this reduces the disease development. Sensitization with guinea pig EP causes an *in vit* cross-reactivity to bovine EP and the reciprocal cross-reactivity probably also exists. In rabbits, this cross-reactivity in both directions was clear-cut. LNC from Lewis rats challenged with guinea pig EP in FCA, with the higher amount of mycobacterium, showed *in vitro* responses to tested peptides of this EP the amino acid sequences of these were 1–42, 43–88, 89–169 and HNB-89-169 (89–169 blocked at the tryptophan-residue by hydroxynitrobenzylation)

Key words: Cell-mediated immune response rats basic proteins of myelin guinea pig cattle

J. Lindh, Tornblad Institute, Biskopsgatan 7 S-223 62 Lund, Sweden.

Received 26 76 Accepted 8 xii.76

Experimental allergic encephalomyelitis (EAE) is readily produced in many species by the injection of tissue from the central nervous system, the basic protein of myelin (also called encephalitogenic protein or EP) or certain peptides derived from EP emulsified in Freund's complete adjuvant (FCA). It is generally agreed that cell-mediated immunity is responsible for the disease.

Evidence has accumulated showing that there can often be a dissociation between the cellular immune response to EP or some of its peptides and disease development. Challenge with EP peptides that do not produce EAE can give a clear-cut cellular immune response measurable by various techniques (*Spitler et al.* 1972, 1975 *Webb et al.* 1973 *Williams & Moore* 1973 *Vandenbark & Hinrichs* 1974 *Hashim et al.* 1976). The guinea pig EP is

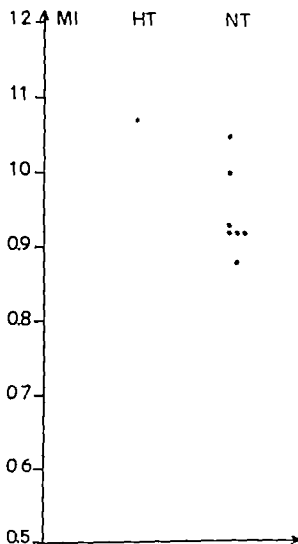


Fig 1 Abscissa Arterial protein concentration in the culture chambers (50 µg protein/ml in the experimental group and the control group) Ordinate Migration index (MI) HT indicates the results from the borderline hypertensive patients and NT the results from the normotensive control persons The two groups of migration indices are significantly different, P<0 005

strated a delayed type of hypersensitivity against arterial wall components in patients suffering from *severe* essential hypertension (*Olsen* 1974) The question is still open as to whether delayed hypersensitivity in essential hypertensive patients is a secondary phenomenon to the hypertensive damage of the arterial vessels, or whether it is an important or indeed a causal factor in the pathogenesis of some cases of essential hypertension Previous studies on different types of arterial vessel damage, including that found in cases of secondary hypertension, showed that such damage was insufficient to produce cellular antibodies as measured by the leucocyte migration technique (*Olsen* 1974) The present results support the view that delayed hypersensitivity directed against arterial wall components might be an important pathogenetic factor in the development of the arterial hypertension in some cases of essential hypertension, since it is possible to demonstrate the hypersensitivity even at a very early stage when the hypertension is only borderline.

REFERENCES

Bendixen G & Søborg M A leucocyte migration technique for in vitro detection of cellular (delayed type) hypersensitivity in man. Danish Medical Bulletin *16* 1–6 1969

Gerner Svendsen U. Studies elucidating the importance of thymus on the degree of increased blood pressure and vascular disease in renal hypertensive mice A comparison of the disease in nude and haired littermates. Acta path. microbiol scand. Sect. A, *83* 568–572, 1975.

Gerner Svendsen U The role of thymus for the development and prognosis of hypertension and hypertensive vascular disease in mice following renal infarction. Acta path. microbiol. scand. Sect. A, *84* 235–243 1976.

Julius S Ellis N C Pascual A I Matice M Hansson L Hunyor S N & Sandler L N Home blood pressure determination. JAMA *229* 663–666 1974

Julius S & Esler M Autonomic nervous cardiovascular regulation in borderline hypertension. Amer J Cardiology *36* 685–696, 1975.

Olsen F Evidence for an immunological factor in the hypertensive vascular disease. Acta path microbiol scand. Sect. A, *79* 22–26 1971

Olsen F Delayed hypersensitivity and essential hypertension Delayed hypersensitivity against arterial wall components as a pathogenetic factor in essential hypertension. Acta path. microbiol. scand. Sect. B, *82* 448–450 1974

Olsen F & Loft B Delayed hypersensitivity directed against arterial antigens in the hypertensive disease in man. Acta path. microbiol scand. Sect. A, *81* 498–500 1973

Rojo-Ortega J M Yeghiayan E. & Genest J The thymus of spontaneously hypertensive rats Light and electron-microscopic studies. Clin Science and Mol. Med. *45* 141s 144s, 1973.

Søborg, M & Bendixen G Human lymphocyte migration as a parameter of hypersensitivity Acta med. scand. *181* 247–255 1967

Acta path. microbiol. scand. Sect. C, 85 199–206, 1977

CELL-MEDIATED IMMUNE RESPONSE TO GUINEA PIG AND BOVINE BASIC PROTEINS OF MYELIN IN LEWIS AND PVG RATS AND THEIR HYBRIDS

Johannes Lindh

Tornblad Institute, University of Lund, Lund, Sweden

Lindh, J. Cell-mediated immune response to guinea pig and bovine basic proteins of myelin in Lewis and PVG rats and their hybrids. Acta path. microbiol. scand. Sect. C, 85 199–206, 1977

The *in vitro* cell-mediated immune response of rat lymph node cells (LNC) to guinea pig and bovine encephalitogenic protein (EP) has been studied with a LNC transformation test. LNC were obtained from either Lewis, PVG or F (Lewis x PVG) rats 28 days after challenge with guinea pig or bovine EP in Freund' complete adjuvant (FCA) of different mycobacterium content. N differences between the strains or their hybrids in LNC response could be shown if stimulated with sensitizing EP despite the great differences in capacity for disease development in those animals. Nor did fivefold lowering of the amount of mycobacterium content in FCA, when guinea pig EP was used t challenge, lessen the vitro response although this reduces the disease development. Sensitization with guinea pig EP causes an *in vitro* cross-reactivity to bovine EP and the reciprocal cross-reactivity probably also exists. In rabbits, this cross-reactivity in both directions was clear-cut. LNC from Lewis rats challenged with guinea pig EP in FCA, with the higher amount of mycobacterium, showed vitro responses to tested peptides of this EP the amino acid sequences of these were 1–42 43–88, 89–169 and HNB-89-169 (89–169 blocked at the tryptophan-residue by hydroxynitrobenzylation)

Key words Cell-mediated immune response rats basic proteins of myelin guinea pig cattle.

J. Lindh, Tornblad Institute, Biskopsgatan 7 S-223 62 Lund, Sweden.

Received 26.x.76 Accepted 8.xii.76

Experimental allergic encephalomyelitis (EAE) is readily produced in many species by the injection of tissue from the central nervous system, the basic protein of myelin (also called encephalitogenic protein or EP) or certain peptides derived from EP emulsified in Freund's complete adjuvant (FCA). It is generally agreed that cell-mediated immunity is responsible for the disease.

Evidence has accumulated showing that there can often be a dissociation between the cellular immune response to EP or some of its peptides and disease development. Challenge with EP peptides that do not produce EAE can give a clear-cut cellular immune response measurable by various techniques (*Spitler et al.* 1972, 1975 *Webb et al* 1973 *Williams & Moore* 1973 *Vandenbark & Hinrichs* 1974 *Hashim et al.* 1976) The guinea pig EP is

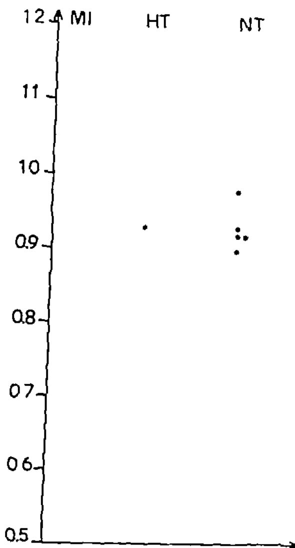


Fig 1 Abscissa Arterial protein concentration in the culture chambers (50 μg protein/ml in the experimental group and the control group) Ordinate Migration index (MI) HT indicates the results from the borderline hypertensive patients and NT the results from the normotensive control persons. The two groups of migration indices are significantly different, $P < 0.005$

strated a delayed type of hypersensitivity against arterial wall components in patients suffering from *severe* essential hypertension (*Olsen* 1974) The question is still open as to whether delayed hypersensitivity in essential hypertensive patients is a secondary phenomenon to the hypertensive damage of the arterial vessels, or whether it is an important or indeed a causal factor in the pathogenesis of some cases of essential hypertension. Previous studies on different types of arterial vessel damage, including that found in cases of secondary hypertension, showed that such damage was insufficient to produce cellular antibodies as measured by the leucocyte migration technique (*Olsen* 1974) The present results support the view that delayed hypersensitivity directed against arterial wall components might be an important pathogenetic factor in the development of the arterial hypertension in some cases of essential hypertension since it is possible to demonstrate the hypersensitivity even at a very early stage when the hypertension is only borderline.

REFERENCES

Bendixen G & Søborg M A leucocyte migration technique for in vitro detection of cellular (delayed type) hypersensitivity in man. Danish Medical Bulletin *16* 1–6, 1969

Gerner Svendsen U Studies elucidating the importance of thymus on the degree of increased blood pressure and vascular disease in renal hypertensive mice A comparison of the disease in nude and haired littermates. Acta path. microbiol scand. Sect. A, *83* 568–572 1975.

Gerner Svendsen U The role of thymus for the development and prognosis of hypertension and hypertensive vascular disease in mice following renal infarction. Acta path. microbiol. scand Sect. A, *84* 235–243 1976

Julius S Ellis N C Pascual, A I Matice M Hansson L. Hunyor S N & Sandler L N Home blood pressure determination. JAMA *229* 663–666 1974

Julius S & Esler M Autonomic nervous cardiovascular regulation in borderline hypertension Amer J Cardiology *36* 685–696 1975.

Olsen F Evidence for an immunological factor in the hypertensive vascular disease Acta path microbiol. scand. Sect. A, *79* 22–26 1971

Olsen F Delayed hypersensitivity and essential hypertension. Delayed hypersensitivity against arterial wall components as a pathogenetic factor in essential hypertension. Acta path. microbiol. scand. Sect. B *82* 448–450, 1974

Olsen F & Loft B. Delayed hypersensitivity directed against arterial antigens in the hypertensive disease in man. Acta path. microbiol. scand. Sect. A, *81* 498–500 1973

Rojo-Ortega J M Yeghiayan E & Genest J The thymus of spontaneously hypertensive rats. Light and electron-microscopic studies. Clin Science and Mol Med *45* 141s–144s, 1973.

Søborg M & Bendixen G Human lymphocyte migration as a parameter of hypersensitivity Acta med scand *181* 247–255 1967

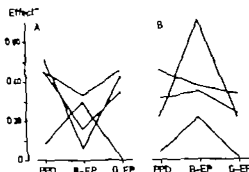


Fig. 1. "Effect"-values calculated as (log c.p. 20 minutes for "S" – log c.p. 20 minutes for NaCl) where "S" stands for the stimulatory agent: PPD, B-EP (bovine EP) or G-EP (guinea pig EP) and NaCl is the saline control. A: an experiment comprising four Lewis rats, 28 days earlier challenged with guinea pig EP in FCA 5X. B: four PVG rats challenged with bovine EP in FCA 5X.

and washed once with cold TCA and once with cold absolute alcohol. The residue was dissolved in 1 ml Soluene 350 for one hour. This was then dissolved in 14 ml of scintillation fluid (500 mg dimethyl-POPOP and 5 g PPO in 1000 ml of toluene) and radioactivity was assayed by counting in Packard 3310 liquid scintillator for two 10-minute-periods. After background subtraction, radioactivity was expressed as number of counts registered (c.p. 20 min). This was logarithmated (log_{10}) and the mean of triplet or quadruplet was calculated.

RESULTS

Stimulation in Vitro with Sensitizing EP

Figure 1 exemplifies the results recorded at the *in vitro* stimulations with PPD, bovine EP or guinea pig EP. The results are expressed as "effect"-values calculated as difference between uptake in cultures with antigen added and uptake in cultures with saline added. LNC were used from different strains (or hybrids) 28 days after immunization with either bovine EP or guinea pig EP. PPD usually gave the same stimulation as did the EP used at challenge, whereas the other EP usually gave a lower stimulation. A complicating factor was the high and variable "background" uptake in control cultures (without antigen added). The magnitude of the further stimulation obtained with the antigen also varied considerably between different rats even after identical immunization. In order to make possible a comparison of the effect of different antigens, different strains and different immunization procedures, a statistical technique had to be applied which permitted a compensation for this variability.

Figure 2 shows the results from one series of experiments. LNC from 38 Lewis rats immunized with guinea pig EP in FCA 5X and stimulated with guinea pig EP *in vitro*. The 1:1 line shows the expected regression at no stimulative effect of EP and the hatched line represents the regression line obtained with the least squares method. The sum of squares around this line is 2.6264 (36 d.f.) and around the $\hat{Y} = X$ line 4.4927 (37 d.f.). The former thus eliminates 1.8663 (1 d.f.) which is highly significant ($F = 25.6$, $P < 0.001$). On the other hand, the fitted line is probably not parallel to the $Y = X$ line as the regres-

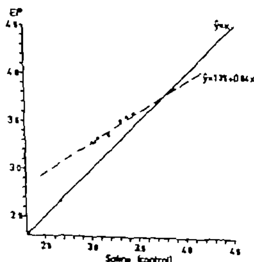


Fig. 2. Log c.p. 20 minutes in LNC cultures from 38 Lewis rats, 28 days earlier challenged with guinea pig EP in FCA 5X. The abscissa gives the uptake values in the saline controls, and the ordinate gives corresponding values in cultures stimulated with guinea pig EP. The dashed line shows the best fitting regression line, the whole line shows the $\hat{Y} = X$ line, indicating no stimulation.

highly encephalitogenic in the Lewis rat compared with the much lower encephalitogenicity of bovine EP (*McFarlin et al* 1973 *Vandenbark & Hinrichs* 1974 *Martenson et al* 1975 a, b), but both antigens will evoke a cell mediated immune response demonstrable with *in vitro* techniques (*Vandenbark & Hinrichs* 1974)

Different species or strains differ in susceptibility to EAE thus the Lewis strain is very susceptible, whereas PVG and BN are rather resistant (*Hughes & Stedronska* 1973 *Williams & Moore* 1973 *McFarlin et al* 1975 a *Lindh* 1977) *McFarlin et al.* (1975 a b) found that the low-responder strain BN mounted a much lower cell mediated response to the sensitizing antigen than did the susceptible Lewis rat.

The present study investigated the possible correlation between susceptibility to EAE in two strains of rats (one highly and one weakly susceptible) and *in vitro* reactivity to EP using one EP which is strongly encephalitogenic in rats (guinea pig EP) and one which is weakly encephalitogenic in rats (bovine EP) In order to further identify those regions of the guinea pig EP that take part in the *in vitro* reaction different peptides derived from EP were also studied.

MATERIAL AND METHODS

This material was previously presented (*Lindh* 1977) It was then evaluated for the EAE development therefore it is only briefly summarized and supplemented to some extent.

Antigens Bovine and guinea pig EP were prepared according to previously described principles (*Bergstrand* 1971) Peptide derivatives (regions 1–42 43–88, 89–169 and HNB-89-169 the latter prepared by chemical blocking of the tryptophan residue (residue 115) of peptide 89–169 with 2 hydroxy 5-nitrobenzylbromide) (numbering according to the revised amino acid sequence of bovine EP see *Brostoff et al.* 1974) were prepared and characterized according to previously described principles for preparation of the corresponding peptides derived from bovine EP see *Bergstrand* (1971 1973) All antigenic materials were kindly supplied by Dr *H Bergstrand*

Immunization. Each rat was immunized with an antigenic emulsion 0.05 ml in each hind paw The emulsion was composed of equal parts of a saline solution of the EP and of FCA. The total amount of EP/rat was always 50 µg. When FCA 5% was used, each rat received 125 µg, and when FCA 1% was used, each rat received 25 µg of *Mycobacterium butyricum*.

Rats 38 Lewis rats were challenged with guinea pig EP and FCA 5%, 16 with guinea pig EP in FCA 1% and 9* with bovine EP in FCA 5%. 11 PVG rats were challenged with guinea pig EP and FCA 5% and 13 with bovine EP and FCA 5%. 8 F_1 (Lewis x PVG) rats were challenged with guinea pig EP and FCA 5%, 3 with guinea pig EP and FCA 1%, and 4 with bovine EP and FCA 5%. A further 10 rats were used as controls and were injected with FCA only (in order to obtain lymph node enlargement) After 28 days, the regional lymph nodes (popliteal fossa were excised on both sides.

Rabbits. These were injected in both hind paws with antigen solutions emulsified with an equal volume of FCA 1% 0 05 ml in one toe pad of each hind paw Three rabbits received guinea pig EP and three bovine EP The total amount of EP was 50 µg and of *Mycobacterium butyricum* 25 µg. The regional lymph node was excised on one side on day 11 after immunization and on the other side on day 17 after immunization and prepared for further investigation.

Lymphocyte cultures The excised lymph nodes were trimmed and washed with Parker 199, and a cell suspension was prepared and filtered through gauze Cultures were set up with a cell count of 2 × 10^6 cells in 1.5 ml of a medium consisting of 20 per cent rat serum in Parker 199 with antibiotics (50 IU Na-benzylpenicillin/ml medium and 50 µg streptomycin sulphate/ml medium) and heparin (25 IU/ml medium)

To the cultures was further added 0 1 ml/tube of either saline purified protein derivative of tuberculin (PPD) (Statens Seruminstitut, Copenhagen, Denmark) bovine EP or guinea pig EP (of all antigens 50 µg/tube) The lymphocytes from some rats challenged with guinea pig EP in FCA 5% were also tested with one of the following peptides of the guinea pig EP sequence 1–42, 43–88, 89–169 or HNB-89-169 all tested in amounts equimolar to that of the guinea pig EP

The cultures were set up in triplicate or quadruplicate according to availability of cells. Incubation was at 37 C for three days. At the end of the culture period ^{3}H-methyl thymidine (^{3}H TdR Schwarz/Mann, 1 9 Ci/mmol) was added to a final concentration of 1 µCi/ml and incubation was continued for one hour The cells were then spun down and washed once with cold phosphate-buffered saline extracted for 30 minutes with cold 5 per cent trichloroacetic acid (TCA)

---

* 4 of these 9 were not included in the previous report by *Lindh* (1977)

TABLE 2. *Co-variance Analysis of Response in vitro to Added Guinea Pig or Bovine EP in LNC from Rats of Different Genotypes Immunized with the other EP and FCA of Different Mycobacterium Content*

Groups and sources of variation	d.f.	sum of residual squares	co-variance	F	significance
Immunization with guinea pig EP + FCA 5X					
Between genotypes	2	0.0707	0.0354	1.01	NS
Within genotypes	56	1.9612	0.0350		
Immunization with bovine EP + FCA 5X					
Between genotypes	2	0.2179	0.1090	2.50	NS
Within genotypes	21	0.9162	0.0436		
Immunization with guinea pig EP + FCA 5X or 1X all genotypes					
Between mycobact. amounts	1	0.0125	0.0125	<1	NS
Within ditto	76	2.6672	0.0351		

Genotypes Lewis, PVG, and F (Lewis x PVG)
Variance ratio = F

between uptake in culture with antigen and culture with saline. X_e = uptake obtained with the other EP

$$Y = a + k \cdot X + k_e \cdot X_e$$

When all 80 rats immunized with guinea pig EP were treated as one group the equation obtained was

$$Y = 0.06 + 0.98 X + 0.50 X_e$$

The Y value is thus practically equal to the control uptake + half of the further stimulation (= "effect") obtained with the antigen used for immunization. The k_e coefficient differs significantly both from 0 ($t = 4.13$, $P < 0.001$) and from 1 ($t = 4.06$, $P < 0.001$). A very similar equation was obtained when LNC from 26 animals injected with bovine EP was studied

$$Y = -0.12 + 1.03 X + 0.49 X_e$$

Again, the EP used for immunization gives an "effect" amounting to approx. twice that obtained with the other EP.

A co-variance analysis can be performed on this material, similar to that presented above, to test whether differences are seen between strains or between amounts of mycobacterium used. No such differences could be demonstrated (Table 2).

The relatively insensitive rat system with its great sources of variation necessitates this complicated statistical analysis. Rabbits were used in order to further study the "cross-reactivity" in the LNC stimulation test. The back-ground uptake is low in this system, and the stimulation obtained is marked and not dependent on control value variations (cf. *Bergstrand & Källén* 1973 a). When six LNC tests were made from three rabbits injected with guinea pig EP, bovine EP could stimulate the cells on average to 56 per cent of the stimulation obtained with the sensitizing EP. In the opposite experiment, guinea pig EP could stimulate the cells to 82 per cent of that obtained with the bovine EP used for immunization.

Stimulative Ability of Peptides Derived from Guinea Pig EP

In an effort to localize the sites in the guinea pig EP that are responsible for the stimulation *in vitro* four peptides derived

TABLE 1 *Co-variance Analysis of Response* in vitro *to Added Guinea Pig or Bovine EP in LNC from Rats of Different Genotypes Immunized with the same EP and FCA of Different Mycobacterium Content*

Groups and sources of variation	d.f	sum of residual squares	co-variance	F	significance
Immunization with guinea pig EP + FCA 5X					
Between genotypes	2	0 0869	0 0435	<1	NS
Within genotypes	57	5 6601	0.0642		
Immunization with bovine EP + FCA 5X					
Between genotypes	2	0.2977	0 1489	2.19	NS
Within genotypes	22	1 4970	0 0680		
Immunization with guinea pig EP + FCA 5X or 1X all genotypes					
Between mycobact. amounts	1	0 1814	0 1814	3.00	NS
Within ditto	77	4.6554	0.0605		

Genotypes Lewis, PVG and F_1 (Lewis x PVG)
Variance ratio = F

sion coefficient is 0 64 ± 0 15 and thus probably differs from 1 t = 2 4 0 05>P>0 01 The interpretation of this is that the actual response to guinea pig EP *in vitro* varies with the uptake in the control cultures high control cultures apparently do not permit any further stimulation

When different strains of rats or different immunization procedures were compared from the standpoint of *in vitro* reactivity of the LNC to (sensitizing) antigen addition a co-variance analysis was made with the aid of regressions of the type illustrated in Fig 2 Table 1 presents the results The analysis shows that the different groups of rats (different strains different amounts of mycobacterium) challenged with guinea pig or bovine EP in FCA do not differ significantly

If all 80 rats immunized with guinea pig EP (irrespective of strain or amount of mycobacterium) are tested as one group the best fitting regression line will be Y = 1 45 + 0 60 X. The corresponding line for the 26 rats immunized with bovine EP is Y = 1.35 + 0 62 X. The two lines are thus practically identical. In order to ascertain that the reactivity to EP is not unspecific lymph nodes from the rats injected with FCA only were studied in a similar way No reactivity was noted mean effect' value with guinea pig EP was -0 06 and with bovine EP +0.03. Regression analysis performed as above confirms the absence of response.

Cross reactivity between Guinea Pig and Bovine EP

So far the response to the EP used at immunization has been studied and no difference has been found irrespective of strain or amount of mycobacterium used Next, the reactivity to the other EP was studied LNC from rats immunized with guinea pig EP were stimulated with bovine EP and vice versa. Then the statistical problem becomes further complicated. A comparison must be made between two registered variates thymidine uptake in cultures stimulated with guinea pig EP and that in cultures stimulated with bovine EP and both variates depend on the uptake in the control cultures. The relation was studied as a multiple regression between the three variates X_1 = control uptake X_2 = "effect" value obtained with the EP used for immunization and calculated as difference

indicates that the cell-mediated response which is measured *in vitro* might have a specificity to many different regions of EP that are thought to be only weakly encephalitogenic in the rat (see review by *Bergstrand* 1977) Similarly a strong cell-mediated reactivity could be demonstrated after immunization with bovine EP and yet, this is not, or only weakly encephalitogenic in the rat. A further interesting detail is that a reduction of the amount of mycobacterium content in FCA given markedly influences the incidence and degree of EAE (cf. *Lindh* 1977) but has no demonstrable effect on the *in vitro* response to EP

We observed a partial cross-reactivity between EP of bovine and guinea pig origin both in rats and in rabbits. This apparently goes in both directions and is readily explained by the large identical sequences of amino acid residues in the two proteins *Lan-denbark* & *Hinrichs* (1974) however using LNC transformation techniques or peritoneal exudate cell migration inhibition technique could not demonstrate any significant cross-reactivity between guinea pig and bovine EP The explanation of this difference is not clear but it could be due to differences in experimental designs and EP preparation

This investigation was supported by grants from the *Swedish Medical Research Council* (Project B75-16X-3920) and funds of Medical Faculty Lund, Sweden.

REFERENCES

Bergstrand H Isolation and partial characterization of some proteolytically and chemically derived fragments of bovine encephalitogenic protein. Europ J Biochem. *21* 116–124, 1971

Bergstrand H Localization of antigenic determinants on bovine encephalitogenic protein. Further studies with the macrophage migration inhibition assay in guinea pigs Immunochemistry *10* 611–620 1973

Bergstrand H Review Immunochemistry of encephalitogenic protein I *Atassi, M Z* (Ed.) Immunochemistry of Proteins Plenum Press, New York, 1977

Bergstrand H & *Källén B.* Antigenic determinants on bovine encephalitogenic protein. Localization of regions that induce transformation of lymph node cells from immunized rabbits. Europ. J Immunol. *5* 287 292, 1973 a.

Bergstrand H & *Källén B* Antigenic determinants on bovine encephalitogenic protein Studies in rabbits with derivatives of fragment 1–43 and the lymph node cell transformation test. Neurobiology *3* 246–255 1973 b

Bergstrand H & *Källén B.* On the statistical evaluation of the macrophage migration inhibition assay Scand. J Immunol. 2 173–187 1973 c.

Brostoff S W., Reuter W., Hichens M & *Eylar E H* Specific cleavage of the A1 protein from myelin with cathepsin D J biol. Chem. *249* 559–567 1974

Hashim G A., Sharpe R D Carvalho E F & *Stevens L E.* Suppression and reversal of allergic encephalomyelitis in guinea pigs with non-encephalitogen analogue of the tryptophan region of the myelin basic protein J Immunol. *116* 126–130, 1976.

Hughes R A C & *Stedronska, J* The susceptibility of rat strains to experimental allergic encephalomyelitis. Immunology *24* 879–884 1973

Lindh, J Susceptibility to experimental allergic encephalomyelitis in two strains of rats and their hybrids In press Acta neuropath. 1977

Lisak R. P., Zweiman B., Kies M W & *Driscoll, B.* Experimental allergic encephalomyelitis in resistant and susceptible guinea pigs In vivo and in vitro correlates. J Immunol. *114* 546–549 1975

Martenson R. E., Deibler G E., Kies M W & *Levine S* Comparative studies of guinea pig and bovine myelin basic proteins. Partial characterization of chemically derived fragments and their encephalitogenic activities in Lewis rats. J Neurochem. *24* 173–182, 1975 a.

Martenson R. E., Levine S & *Sowinski R* The location of regions in guinea pig and bovine myelin basic proteins which induce experimental allergic encephalomyelitis in Lewis rats. J Immunol. *114* 592–596 1975 b.

McFarlin D E., Blank S E., Kibler R F., McKneally S & *Shapira R.* Experimental allergic encephalomyelitis in the rat. Response to encephalitogenic proteins and peptides. Science *179* 478–480 1973

McFarlin D E., Hsu S C L, Slemenda, S B., Chou F C-H & *Kibler R. F* The immune response against myelin basic protein in two strains of rat with different genetic capacity to develop experimental allergic encephalomyelitis J exp. Med. *141* 72–81 1975 a.

McFarlin D E., Hsu S C L, Slemenda S B Chou S C-H & *Kibler R. F.* The immune response against an encephalitogenic fragment of guinea pig basic protein in the Lewis and

from the EP were tested on LNC from Lewis rats sensitized with guinea pig EP and FCA 5X. Peptides were tested in concentrations equimolar with that used for guinea pig EP and the effects were calculated according to the method used by *Bergstrand & Kallén* (1973 a b c) and expressed as per cent values of the total effect of EP Table 3 summarizes the results.

TABLE 3 *Stimulatory Effect of Peptides Derived from Guinea Pig EP Compared with the Effect of Intact EP Expressed as Per Cent*

Peptide	"degree" of reactivity (%)	differs from 0 %	differs from 100 %
1–42	82 ± 16	$p < 0.001$	NS
43–88	81 ± 16	$p < 0.001$	NS
89–169	55 ± 16	$p < 0.01$	$p < 0.0?$
HNB-89-169	89 ± 16	$p < 0.001$	NS

Calculations performed according to principles described by *Bergstrand & Källén* (1973 a b c) Differences against 0 and 100 per cent are tested with t tests. Ten Lewis rats challenged with guinea pig EP in FCA 5X 28 days earlier

It is obvious that all three main peptides (1–42 43–88 89–169) show a stimulative capacity and that this is not abolished or even reduced for peptide 89–169 by blocking the tryptophan site with HNB. No "master site" is thus demonstrable which carries the main capacity to stimulate LNC to proliferation.

DISCUSSION

The present report shows that immunization of rats with EP in FCA elicited the same strength of *in vitro* demonstrable cell mediated response irrespective of which of the following three rat genotypes was used the Lewis, the PVG or the F_1 (Lewis x PVG) This contrasts to the findings concerning susceptibility to EAE previously described in the same material (*Lindh* 1977) Thus the Lewis strain was found to be very susceptible to EAE, the PVG strain was rather resistant, and the F_1 (Lewis x PVG) hybrids were intermediate. These results differ from those found by *Williams & Moore* (1973) who demonstrated a different strength of delayed type skin reactivity to EP between highly EAE susceptible Lewis rats and resistant BN rats. *McFarlin et al* (1975 a, b) confirmed this, using *in vitro* techniques lymphocyte transformation and macrophage migration inhibition techniques. The reason for this difference is unclear it might be due to different experimental systems, differences in genetic regulation of responses after challenge for EAE, or perhaps different availability of EP *in vivo* to the attack of presumably sensitized cells. Our results more resemble those reported by *Webb et al* (1973) who studied two strains of guinea pigs, strain 2 and 13, one of which is a high responder and the other a low responder no significant difference was found between them with respect to skin reactivity or in *in vitro* lymphocyte stimulation. After repeated stimulation with EP in FCA, the low responding strain even produced a higher titre of antibody to EP (perhaps indicating a "protective" function) as measured with passive cutaneous anaphylaxis. *Lisak et al* (1975) however found a difference between these two guinea pig strains, as the low responder strain gave a weaker cell-mediated response and also a lower humoral response to the sensitizing antigen, but other techniques were used for measurements. The differences in results could be the effect of differences in experimental design, for instance heterologous respectively homologous EP

The fact that rats immunized with EP but only poorly responding with EAE give a marked cell mediated response as estimated with the lymphocyte transformation technique indicates that a cell-mediated response might develop towards parts of EP that are not encephalitogenic This agrees with observations made on guinea pigs (*Spitler et al.* 1972 1975 *Hashim et al* 1976) and rabbits (*Bergstrand & Källén* 1973 a) Furthermore the analysis of the stimulative effect of different peptides derived from guinea pig EP on LNC from Lewis rats sensitized with such EP

Acta path. microbiol. scand. Sect. C, 85: 207–214, 1977

INTERACTIONS OF IMMUNE COMPLEXES AND PLATELETS IN RABBITS IMMUNIZED WITH HAPTEN CARRIER CONJUGATES

RIITTA KEKOMÄKI*, HANNA LEENA KAUPPINEN*, KARI PENTTINEN** and GUNNAR MYLLYLÄ*

* Finnish Red Cross Blood Transfusion Service, Kivihaantie 7, Helsinki, Finland
** Department of Virology, University of Helsinki, Helsinki, Finland

Kekomäki, R., Kauppinen, H. L., Penttinen, K. & Myllylä, G. Interactions of immune complexes and platelets in rabbits immunized with hapten-carrier conjugates. Acta path. microbiol. scand. Sect. C, 85: 207–214, 1977.

The thrombopenic effects of immune complexes were studied in 32 rabbits immunized actively with aspirin. The test animals were exposed to hapten-protein conjugates with a hapten density ranging from 0.6 to 38, and to pure hapten. The resulting thrombopenia and leucopenia correlated closely with the presence of immune complexes in the serum as detected by the platelet aggregation sedimentation pattern test (PIA). This platelet-aggregation activity sedimented mainly in the 19S fraction. The effects induced by polyvalent antigens where dose-dependent and could be modified by prior injection of aspirin. Expectedly, monovalent antigens were only marginally effective.

Key words: Thrombocytopenia; platelet aggregation; leucopenia; immune complexes; hapten-carrier conjugates; rabbits.

R. Kekomäki, Finnish Red Cross Blood Transfusion Service, Kivihaantie, 7, Helsinki 31, Finland.

Received 20. 77 Accepted 20.I.77

Platelet aggregation induced by hapten-specific antigen-antibody complexes in vitro was studied in detail by *Penttinen et al.* (17). Immune complexes formed with specific antibodies and free hapten or monovalent hapten-protein conjugates are not capable of causing platelet aggregation in vitro. But immune complexes formed by polyvalent antigens are effective. Furthermore, an increase in hapten density from about two to 40 correlates with the decreasing amount of hapten required to induce equivalent aggregating effects.

According to the preliminary observations prompting this study aspirin-protein conjugates can cause thrombocytopenia in rabbits actively immunized with aspirin. A series of experiments was designed to characterize this phenomenon and to relate the platelet aggregating activity induced under controlled circumstances in vivo, to the earlier results (17). The effect of hapten density and of the amount of antigen on the reaction induced with hapten-carrier conjugates was investigated. In particular we attempted to characterize the emergence and disappearance of

Brown Norway strains of rat. J Immunol. *115* 1456–1458, 1975 b

Spitler L. E. Muller C M Fudenberg H H & Eylar E H Experimental allergic encephalomyelitis. Dissociation of cellular immunity to brain protein and disease production. J exp Med. *136* 156–174 1972

Spitler L. E Muller C M & Young J D Experimental allergic encephalomyelitis Study of cellular immunity to the encephalitogenic determinant. Cell. Immunol. *15* 143–151 1975

Vandenbark A A & Hinricks D J Experimental allergic encephalomyelitis and cellular immunity in the Lewis rat. Cell. Immunol. *12* 85–93 1974

Webb C Teitelbaum D., Arnon R. & Sela M Correlation between strain differences in susceptibility to experimental allergic encephalomyelitis and the immune response to encephalitogenic protein in inbred guinea pigs. Immunol. Commun. *2* 185–192, 1973

Williams R. M & Moore M J Linkage of susceptibility to experimental allergic encephalomyelitis to the major histocompatibility locus in the rat. J exp Med. *138* 775–783 1973

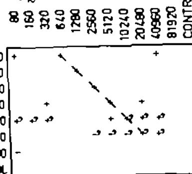


Fig 1 Estimation of the equivalence zone by checker-board titration. For this test animal with body weight of 3.5 kg and plasma volume of about 35 ml/kg, the equivalent dose of aspirin can be extrapolated to be approximately 0.93 mg

feature of the complexes was utilized both to measure antibodies in the immune sera and to detect immune complexes in test sera. The PLA sedimentation pattern test was applied (16). Principally the assay system was as follow. Human platelets from healthy donors were separated according to *Aster & Jandl* (1) washed and suspended to final concentration of 200 000/µl. The procedure was completed within 4–6 hours of bleeding and the platelets were used on the day of preparation. The aspirin-antibodies were measured in checker board titration (13). Aliquots of the antigen and antibody dilutions, both in equal volumes of 0.025 ml, were pipetted onto microplate well and incubated at room temperature for one hour. 0.05 ml of platelet suspension was added and the microplates were left standing at +4 +8 C for 18–20 hours before reading the reactions. When the effect of various reagents was studied, all extra additions were done in standard volume of 0.025 ml. A test setting is depicted in Fig 1. This figure also illustrates the technique of extrapolation of the aspirin equivalence from the results obtained.

The property of serum alone to induce platelet aggregation is defined here as direct platelet aggregating activity. The changes in direct platelet aggregating activity prompted by the exposure to given antigen were used to detect the appearance of immune complexes in test sera.

The sensitivity of individual platelet preparations is known to be variable (14). Thus, direct comparisons were made between the results obtained with one platelet preparation only. Controls including heat-aggregated human IgG rabbit anti-human-thrombocyte antibody rabbit anti-NIP$_{14}$-BSA-complex and fresh human serum were run for each platelet preparation. The sensitivity of the PLA sedimentation pattern test used allows quantification of aggregated human gammaglobulin of the order of 1–4 µg/ml.

Sucrose Gradient Centrifugation

The PLA activity found was characterized with density gradient centrifugation (16). Paul-Bunnel positive serum was used as 19 S reference marker and as 7 S marker rubella positive serum. The sucrose in Tris-buffer did not affect the platelet patterns after 4-fold dilution of the fractions.

RESULTS

Effect of the Hapten Density on the Reactions caused by the Antigen Exposure

Experiments with polyvalent antigens. The injection in 33 experiments of a polyvalent antigen with the hapten density varying from 2.5 to 58 caused invariably a rapidly developing thrombocytopenia and leucopenia. 8–10 minutes after the exposure the amount of circulating cells was approximately one fifth of the original. At two hours, the platelet counts were about 50 per cent of the starting values, whereas the leucocyte counts remained somewhat lower than 50 per cent. A constant slight thrombocytosis was observed on the fifth day of the experiment. The leucocyte counts were normalized within 24 hours of exposure (Fig 2 a)

Injection of the antigen was always followed by an instant appearance of immune complexes in the circulation judging by the marked increase of direct PLA activity. The last serum dilutions that were able to induce platelet aggregation varied in different experiments from 1/40 up to 1/320. Within two hours the concentration of immune complexes declined, but some marginal activity usually persisted. Some rabbits demonstrated a second peak of direct PLA activity in the 5-7-day samples (Fig 3). In none of the non-immunized control rabbits could either cellular changes or alterations in direct PLA activity be observed.

Experiments with monovalent antigens. The hapten density of the antigens employed in these series were 0.6, 0.9, 1.1, 1.5 and 1.6. The ag/ab ratio was near the equivalence.

immune complexes after a single injection of hapten-carrier conjugates

MATERIALS AND METHODS

Antigens

Aspirylchloride (Merck AG Darmstadt, W Germany) was coupled to rabbit agammaserum (RAGS normal rabbit serum was treated at +4 C with 35 per cent aqueous $(NH_4)_2 SO_4$ solution) bovine serum albumin (BSA Sigma AG St. Louis, Mo. USA) human serum albumin (HSA Finnish Red Cross Blood Transfusion Service) and ovalbumin (OA Fluka AG Buchs, Switzerland) according to a modification of the Schotten Baumann reaction (23) The number of aspiryl groups per mole of protein (= hapten density) was determined spectrophotometrically by measuring E_{80}, Asp_{22}RAGS was used for immunization of the test animals (see below)

Eight BSA conjugates (hapten densities ranging from 0 6 to 38) five HSA conjugates (1 1 to 20) and $Asp_{1.4}$OA were used in the experiments (for details of the hapten conjugates, see Table 1) All the antigens used were tested for direct platelet aggregating activity (see below) with consistently negative results.

TABLE 1 *Characteristics of the Aspiryl protein Conjugate Solutions Used in the Study*

	Asp mmol/l	Prot. mg/ml
Asp $_{.4}$BSA	0 05	5 70
$Asp_{0.9}$BSA	0 12	8.74
Asp BSA	0 25	11 10
$Asp_{3.3}$BSA	0 20	5 40
Asp BSA	0.70	3 75
Asp_{10} BSA	1 67	6 25
Asp $_{7}$ BSA	0 62	2 50
Asp_{18} BSA	0 73	2 75
Asp_{38} BSA	2 04	3 60
$Asp_{1.1}$HSA	0 11	6 65
Asp $_{.4}$HSA	0 ?3	10 70
Asp_1 HSA	1 15	8.06
Asp_{13} HSA	0 99	5 30
Asp_{20} HSA	2.71	9.21
$Asp_{1.4}$ OA	0.27	7 85

The concentration of conjugated aspirin in each antigen preparation was smaller than 1×10^{-3} mmol/l. Each antigen was tested for pyrogenicity with a constant monitoring rectal thermometer

Immunization

Thirty two female rabbits were used in the experiments They were 2-3 month old and weighed approximately 2.5 kg Asp_{22}RAGS (content of total nitrogen 0 18 mg/ml) was emulgated with an equal volume of complete Freund's adjuvant. One ml of this mixture was injected i.m. or s.c. seven times at one-week intervals into 30 rabbits (hyperimmunization) Two of the animals were given two weekly injections only (minimal immunization) One week after the last injection the antibody level was measured (see below)

Determination of Antibodies

Ouchterlony's microtechnique gel diffusion was employed as a preliminary method for the detection of aspiryl antibodies. The test was carried out in 1 per cent agarose (L Industrie Biologique Française S.A. Gennevillier France) dissolved in 0 5 M phosphate-buffered saline, pH 7.3 Thereafter the actual titer of antibodies in all of the immune sera was determined by the PLA. test (see below) The highest dilution of the tested sera giving a positive reaction was 1/640 in most of the rabbits, and 1/320 in the others. No evidence was found of antibodies against carrier proteins (RAGS, BSA, HSA, OA) To confirm this finding, some of the immune sera were absorbed chromatographically with Sepharose-BSA (20) As studied by the PLA test and Ouchterlony's double diffusion technique the concentration of aspiryl antibodies was not affected by this treatment, nor were any anti-BSA antibodies found in the eluate. The sera of four non-immunized rabbits were subjected to the same study programme

Experiments in Vivo

In the first series of experiments the test animals were injected with Asp-BSA conjugates (see Table 1) The same animals were used again eight weeks later To avoid the bias caused by carrier-specific antibodies at the second stage Asp-HSA was used. The antigen solution was injected rapidly into the marginal vein of the ear Blood samples were drawn from the opposite ear for the count of platelets and leucocytes (Buerger Tuerk chamber) and for the PLA. test before the injection of antigen, immediately (e.g., within 1-3 minutes) thereafter and then at the time intervals indicated Ten of the animals were bled through a catheter inserted into the deep jugular vein to collect larger blood volumes for analysis of the sera for immune complexes. No measurable dilution of the blood was caused by the sampling The sera were preserved as small samples at -20 C until analyzed.

The non immunized control rabbits were exposed to representative antigens. The subsequent analytical procedure wa as for the test animal

Determ tion of Immune Complexes

Immune complexes can trigger platelet aggregation through the Fc parts of the antibodies. This

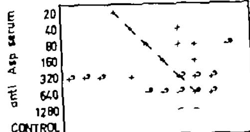


Fig 1 Estimation of the equivalence zone by checker-board titration. For this test animal with body weight of 3.5 kg and plasma volume of about 35 ml/kg, the equivalent dose of asparin can be extrapolated to be approximately 0.95 mg.

feature of the complexes was utilized both to measure antibodies in the immune sera and to detect immune complexes in test sera. The PLA sedimentation pattern test was applied (16). Principally the assay system was as follow. Human platelets from healthy donors were separated according to *Aster & Jandl* (1) washed and suspended to final concentration of 200,000/µl. The procedure was completed within 4–6 hours of bleeding and the platelets were used on the day of preparation. The aspirin-antibodies were measured in checker board titration (13). Aliquots of the antigen and antibody dilutions, both in equal volumes of 0.025 ml, were pipetted onto microplate well and incubated at room temperature for one hour. 0.05 ml of platelet suspension was added and the microplates were left standing at +4 +6 C for 18–20 hours before reading the reactions. When the effect of various reagents was studied, all extra additions were done in standard volume of 0.025 ml. A test setting is depicted in Fig. 1. This figure also illustrates the technique of extrapolation of the aspirin equivalence from the results obtained.

The property of serum alone to induce platelet aggregation is defined here as "direct" platelet aggregating activity. The changes in direct platelet aggregating activity prompted by the exposure to given antigen were used to detect the appearance of immune complexes in test sera.

The sensitivity of individual platelet preparations is known to be variable (14). Thus, direct comparisons were made between the results obtained with one platelet preparation only. Control including heat-aggregated human IgG rabbit anti-human-trombocyte antibody rabbit anti-NIP$_{14}$ BSA-complex and fresh human serum were run for each platelet preparation. The sensitivity of the PLA sedimentation pattern test used allows quantification of aggregated human gammaglobulin of the order of 1–4 µg/ml.

Sucrose Gradient Centrifugation

The PLA activity found was characterized with density gradient centrifugation (16). Paul-Bunnel positive serum was used as 19 S reference marker and as 7 S marker rubella positive serum. The sucrose in Tris-buffer did not affect the platelet patterns after 4-fold dilution of the fractions.

RESULTS

Effect of the Hapten Density on the Reactions caused by the Antigen Exposure

Experiments with polyvalent antigens. The injection in 33 experiments of a polyvalent antigen with the hapten density varying from 2.5 to 38 caused invariably a rapidly developing thrombocytopenia and leucopenia. 8–10 minutes after the exposure the amount of circulating cells was approximately one-fifth of the original. At two hours, the platelet counts were about 50 per cent of the starting values, whereas the leucocyte counts remained somewhat lower than 50 per cent. A constant slight thrombocytosis was observed on the fifth day of the experiment. The leucocyte counts were normalized within 24 hours of exposure (Fig 2 a).

Injection of the antigen was always followed by an instant appearance of immune complexes in the circulation judging by the marked increase of direct PLA activity. The last serum dilutions that were able to induce platelet aggregation varied in different experiments from 1/40 up to 1/320. Within two hours the concentration of immune complexes declined, but some marginal activity usually persisted. Some rabbits demonstrated a second peak of direct PLA activity in the 5-7-day samples (Fig 3). In none of the non-immunized control rabbits could either cellular changes or alterations in direct PLA activity be observed.

Experiments with monovalent antigens. The hapten density of the antigens employed in these series were 0.6 0.9 1.1 1.5 and 1.6. The ag/ab ratio was near the equivalence.

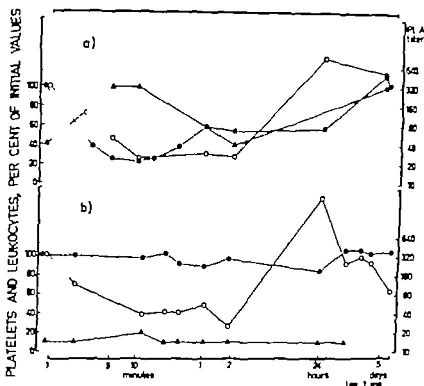


Fig 2 a. Representative changes in rabbit blood platelet and leucocyte counts and in the PLA titer induced by an i.v. injection of an equivalent dose of polyvalent Asp-BSA antigen.
Fig 2 b The effects of a similar injection of a monovalent Asp-BSA antigen.

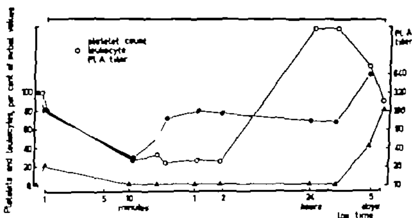


Fig 3 An experiment demonstrating a second rise in Pl A activity appearing 5 days after exposure to a polyvalent Asp-BSA antigen.

Correspondingly the amount of injected aspirin was approximately 0 25 mg/rabbit

The injection of monovalent antigens led systematically to leucopenia of varying degrees, whereas an effect on the platelet counts was rarely seen (Fig 2 b) Hardly detectable increments in direct PlA activity occurred simultaneously Qualitatively these changes were similar to those induced by polyvalent antigens. This phenomenon can presumably be attributed to the presence of minimum amounts of polyvalent conjugates in these preparations.

Experiments with unconjugated hapten All the parameters monitored remained unchanged when the immunized rabbits were injected with an equivalent dose or equivalent dose × 10^2 of aspirin in physiological saline

The Effect of the Amount of Antigen on the Reaction Induced with Polyvalent Hapten Protein Conjugates

With the amount of antigen decreasing the changes in direct PlA activity and cell counts diminished (Fig 4) The smallest amount of aspirin potent enough to induce alterations in the measure parameters was 0 6 µg/rabbit

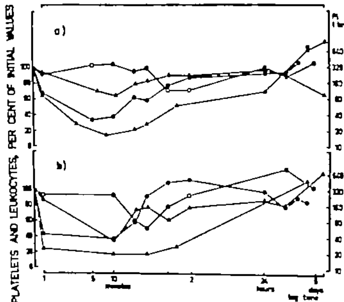


Fig. 4 The effect of the amount of polyvalent antigen on blood platelet (a) and leucocyte (b) counts. Four experiments. Amounts of antigen, expressed as absolute aspirin

open circles = 0.06 μg
open triangles = 0.6 „
solid circles = 6.0
solid triangles 300

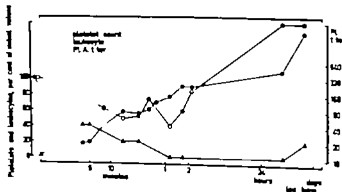


Fig. 5 An attempt to demonstrate hapten inhibition. The test animal was injected with 7 mg of acetosalicylic acid (a 10-fold dose of equivalence) 1 minute prior to injection of polyvalent antigen.

or 1/1000 of the equivalence. One-tenth of this dose was without effect. On the other hand, exceeding the equivalent dose × 10 of antigen did not change the outcome of the experiment either in the hyperimmunized or in the two minimally immunized rabbits.

Attempts to Demonstrate Hapten Inhibition

A prior injection of an excess (4–28 × equivalence ca 1–7 mg/rabbit) of free aspirin did not inhibit the reaction. However when the largest dose (7 mg) of free hapten was used, the platelet and leucocyte counts attained their normal values in a clearly shorter time than without this pretreatment (Fig 5) In vitro control experiments indicated that pretreatment with aspiryl-chloride (0.1 mg/ml) of the platelets used in the PLA test did not affect their reactivity either for the aspiryl- or for the NIP-[illegible]-carrier complexes.

Studies on the Circulating Immune Complexes

Sera drawn immediately after the polyvalent antigen exposure presented the highest direct PLA activity. Eight such samples were subjected to density gradient centrifugation. All these sera showed direct PLA activity sedi-

menting as fast or faster than the 19S marker (Fig 6) For further characterization of this zone of activity, purified rheumatoid factor (Cryo-IgM a generous gift from Dr *O Wager*) was added to the incubation mixture. This treatment is known to diminish the immune complex induced aggregation of platelets (11 21) In the present series, significant decrements in PLA. titers were recorded Some sera showed direct PLA activity at the 7S marker as well (Fig 6)

In addition to the samples obtained immediately after exposure, four serum samples taken five days later and demonstrating PLA. activity were analyzed similarly In two of them all the aggregating activity located now at the 7S region This activity was also inhibited by rheumatoid factor (Fig 7) In the other PLA. activity sedimented to three regions of fractions and was found 1 in the region of 19S 2. in the region of 7S and 3 in the pellet.

The Fc mediated platelet IgG interactions can be blocked by monomeric (native) IgG (18) To test the direct PLA. activity fresh serum was added to the platelet suspension prior to the incubation with test sera The PLA activity appearing in various phases during the post-exposure period was invariably abolished by this pretreatment.

One immune serum drawn prior to the exposure was fractioned with density gradient centrifugation Expectedly the addition of a polyvalent antigen to the fractions induced direct PLA. activity in the 7S region where the IgG molecules are sedimented

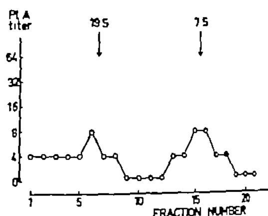


Fig 6 Sucrose density gradient fractionation of a serum drawn immediately after exposure to polyvalent antigen expressed as direct PLA activity PLA. titer of the serum 320

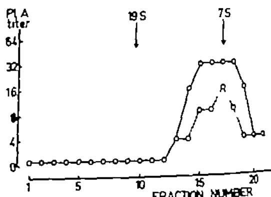


Fig 7 Sucrose density gradient fractionation of serum demonstrating the second rise in direct PLA activity (solid line) PLA. activity of the same fractions after treatment with rheumatoid factor (broken line) PLA. titer of the serum 320

DISCUSSION

The in vivo hapten-conjugate antibody interactions seen in the present series parallel in many respects the information obtained earlier from in vitro experiments (17)

The exposure of hyperimmune rabbits to polyvalent hapten conjugates caused an immediate increment in the direct PLA. activity of the serum. Simultaneously with this occurrence, the blood platelet and leucocyte counts diminished temporarily The described reaction was reproducible and dose-dependent, as suggested by *Israels et al.* (9) (Fig 4) Monovalent antigens prompted only slight changes in these parameters.

Efforts to demonstrate hapten inhibition gave somewhat equivocal results. This may be due to a suboptimal choice of hapten aspirin is distinguished by its direct stabilizing action on thrombocyte membrane which may render the cells less apt to aggregation (6) However the results of control experiments run in vitro were at variance with this fact.

The following data corroborating the identity of direct PLA. activity and circulating immune complexes were collected

1. the immediate cellular changes were reciprocal to those observed in direct PLA. titer and explainable by the Fc mediated immune complex cell interactions (9 12, 13)
2. in density gradient centrifugations the PLA active fractions sedimented as fast as or faster than the 19S marker
3. this PLA activity could be markedly extinguished either by a pretreatment of test platelets by fresh human serum because monomeric IgG inhibits competitively the reaction on the Fc receptors, or by the addition of Cryo-IgM to the incubation system before platelets. Here the factor acts as steric hindrance of the platelet IgG interactions (21)
4. the direct PLA. activity tested always with two different platelet preparations, varied pari passu with the control samples with in vitro prepared NIP-immune complexes with rabbit antiserum (13)

No exact data can so far be provided on the fate of platelets disappearing from the circulation. A fraction of platelets "sensitized" by immune complexes is extracted. Within two hours of exposure, however the thrombocytopenia reverses significantly to yield levels approximately half as high as the original ones. The following slight but consistent thrombocytosis suggests real disintegration of a fraction of the circulating platelet pool (13)

Different mechanisms have been proposed for the immune complex-induced platelet alterations in rabbits (7 8, 3) complement mediated platelet injury has been shown to occur both in vivo and in vitro (8) but complement-independent systems have also been described, e.g., the direct interaction of platelets with immune complexes (4 7 19 22) and the complement-independent, leucocyte dependent mechanism (2) Furthermore, species-specific differences are known to exist in the receptor structures of human and rabbit platelets. Because human platelets were used in the PLA. test in this study the direct PLA. activity found cannot be expected to reflect the in vivo sequestration phenomenon of rabbit platelets in all detail. Due to its sensitivity however the technique is very suitable for experimental work. The smallest amount of hyperimmune antibodies which can be recorded by the PLA. test is approx. 1 µg/ml (17) Reportedly the PLA. test detects at least IgG-containing immune complexes of rather great size (13)

Studied in rabbits, the half-life of the preformed immune complexes is approximately 20 min for the complexes with a sedimentation ratio of 19S or greater whereas the smaller complexes live somewhat longer (10) This parallels the results of the present series, provided that the formation of immune complexes is limited to the first hour following exposure. The immune complexes are eliminated by a primary formation of lattices, which are extracted secondarily by filtration from the circulation. Mainly immune complexes sedimenting as fast as or faster than 19 S marker are shown in the endothelium of veins (5) The rate of lattice formation and also that of elimination is a function of the ag/ab ratio, antigen excess leading to a slower rate of immune complex disappearance.

The inconsistent presentation of PLA. activity in 7S fraction remains enigmatic. Because of its small size, it can hardly be an intact complex formed in the antigen-antibody reaction studied. Carrier-induced reaction (serum sickness) appears to be excluded by the unchangeable PLA. activity after the absorption of the serum with Sepharose BSA. Complement-employing systems for immune complex detection would hardly solve the problem, because small complexes escape detection by these techniques, too. The possibility remains that this direct PLA. activity is due to an antibody reactive with platelets.

This study was aided in part by a grant from *Sigrid Jusélius Foundation*.

REFERENCES

1 *Aster R. H & Jandl, J H.*, Platelet sequestration in man. I Methods. J clin. Invest. *43* 843–855 1964

2 *Benveniste J & Henson P M* Leucocyte-dependent mechanism of histamine release from rabbit platelets transfer of responsible antibody Fedn Proc. Fedn Am. Socs exp Biol *30* 654 1971

3 *Benveniste J Henson P M & Cochrane C G* Leucocyte-dependent histamine release from rabbit platelets. The role of IgE basophils and a platelet-activating factor J exp. Med. *136* 1356–1377 1972.

4 *Benveniste J* Platelet-activating factor a new mediator of anaphylaxis and immune complex deposition from rabbit and human basophils. Nature (London) *249* 581–582, 1974

5 *Cochrane C G* Mechanisms involved in the deposition of immune complexes in tissues. J exp Med. *134* 75–89 1971

6 *Evans G E. Packham M A Nishizawa, E E. Mustard J F & Murphy E. A* The effect of acetylsalicylic acid on platelet function. J exp Med. *128* 877–894 1968

7 *Henson P M* The role of complement and leucocytes in the immunologic release of vasoactive amines from platelets. Fed Proc. *98* 1721 1969

8. *Henson P M* Mechanisms of release of constituents from rabbit platelets by ag ab complexes and complement. I Lytic and non-lytic reaction. J Immun. *105* 476–489 1970

9 *Israels E D Nisli G Paraskevas P & Israels L. G* Platelet Fc receptor as a mechanism for Ag Ab complex-induced platelet injury Thromb Diath. haemorrh. *29* 434–444 1973

10 *Lightfoot R W Drunn R E. & Christian C I* Properties of soluble immune complexes J Immun *105* 1493–1500 1970

11 *Mowbray J F & Panyananda A* Platelet trombi in rejection of renal allografts. Thromb Diath. haemorrh. Suppl XLV *183–190* 1971

12 *Mueller Eckardt C & Luscher E F* Immune reactions of human platelets. I A comparati e study on the effects on platelets of heterology antiplatelet antiserum antigen-antibody complexes, aggregated gammaglobulin and thrombin. Thromb Diath. haemorrh. *20* 155–167 1968.

13 *Myllylä G.* Aggregation of human blood platelets by immune complexes in the sedimentation pattern test. Scand. J Haemat. Suppl 19 1973 Thesis.

14 *Myllylä G* Role of platelets in immunecomplex induced aggregation of human blood platelets. Annls. Med. exp. Biol. Fenn. *51* 22 33, 1973

15 *Odell T T., Jackson C H & Murphy J R* Platelet recovery after induction of acute thrombocytopenia. Proc. Soc. exp. Biol Med *148* 829–833 1975

16 *Penttinen K & Myllylä G.* Interaction of human blood platelets, viruses and antibodies I Platelet aggregation test with microequipment. Annls Med. exp. Biol. Fenn. *46* 188–192, 1968

17 *Penttinen, K., Myllylä G Mäkelä, O & Lahiri A* Soluble antigen-antibody complexes and platelet aggregation. Acta path. microbiol scand, *77* 309–317 1969

18. *Pfueller L. & Lüscher E. F.* The effects of aggregated immunoglobulins on human platelets in relation to their complement fixing abilities. II Structural requirements of the immunoglobulin. J Immun. *109* 526–532, 1972.

19 *Pfueller S L. & Lüscher E. F* Studies of the mechanisms of the human platelet release reaction induced by immunologic stimuli. I Complement-dependent and complement-independent reactions. J Immun. *112* 1201–1210, 1974

20 *Porath J Axen R & Ernback S* Chemical coupling of proteins to agarose Nature (London) *215* 1491–1492 1967

21 *Wager O., Penttinen K & Myllylä, G* Precipitation of IgG complexes and inhibition of complex induced platelet aggregation by RF active cryoglobulin M component. Scand J clin. Lab. Invest. *29* suppl 122·62, 1972.

22. *Wautier J L. Tobelem G M., Peltier A. P & Caen J P* Evidence for C_1 on human platelets. Haemostasis 2 281–286, 1973

23 *Wicher K Schwartz M Arbesman C. E & Milgrom F* Immunologic studies on aspirin. I Antibodies to aspirylprotein conjugates. J Immun. *101* 342–348 1968.

Acta path. microbiol. scand. Sect. C, 85 215–221 1977

CHARACTERISTICS OF THE PHAGOCYTIC PROCESS ASSESSED BY COULTER COUNTER

K. E. Magnusson, C. Dahlgren, O. Stendahl and T. Sundqvist

Department of Medical Microbiology, The University of Linköping, S-581 85 Linköping, Sweden.

Magnusson, K.-E., Dahlgren, C., Stendahl, O. & Sundqvist, T. Characteristics of the phagocytic process assessed by Coulter Counter. Acta path. microbiol. scand. Sect. C, 85 215–221 1977

The phagocytosis in suspensions of heat-killed yeast cells, *Saccharomyces cerevisiae* by human polymorphonuclear leucocytes was studied *in vitro* by means of an electronic particle counter, the Coulter Counter, and a 100-channel pulse-height analyser, the Channelyzer. The two cell populations were separated from each other electronically by the Channelyzer. Phagocytosis was recorded as disappearance of yeast cells. Concomitantly, aggregation and swelling of the PMN cells were observed, which increased with the concentration of the prey. The process could be inhibited by cytochalasin B and iodoacetamide. With the latter inhibitor, the analysis of the kinetics showed that ingestion, but not adhesion, was affected. The ingestion of yeast cells was augmented on increase of the initial ratio between the number of yeast and PMN cells to around 5:1, but was then reduced on further increase. A ratio of 2:1 and a reaction time of 30 min seemed suitable for studying the phagocytic process. First-order kinetics were obeyed under these circumstances.

Key words: Phagocytic process; characteristics; Coulter Counter.

K.-E. Magnusson, Department of Medical Microbiology, The University of Linköping, S-581 85 Linköping, Sweden.

Received 22.xi.76 Accepted 27.xii.76

The phagocytosis of, for example, microorganisms by polymorphonuclear granulocytes, is generally regarded as a four-stage process, involving recognition, attachment, ingestion and intracellular killing (20, 21). Several methods developed for the *in vitro* assay of phagocytosis are indirect, e.g. O_2-consumption (18) and activation of hexose monophosphate shunt (23), or are restricted to one type of prey, e.g. red oil O (19). Direct observation in the microscope is timeconsuming and subjective.

For the analysis of the interaction of the prey with the phagocyte, it is necessary to know the kinetics of the reaction. Furthermore, if the method is to find application in the laboratory as a test of, for instance, phagocytic capacity of PMN cells from patients, it must be easy to perform and be devoid of operator's variations. Electronic particle counters, such as the Coulter Counter and the Celloscope, are usually used for counting of particles such as blood cells and microorganisms suspended in an electrically conducting medium, usually saline. They have

also been used to study aggregation and agglutination of different particles (15 16 22). With the Coulter Counter up to 10 000 particles can be counted in 13 seconds.

Since the height of the electrical pulse which a particle produces when it passes a narrow capillary depends on the volume the counter has also been used for sizing of particles (2 6). However absolute volume measurements are difficult to achieve since the pulse, besides depending on the volume also depends on the shape (4) and the resistivity of the particle (8 9 11 17).

In order to find optimal conditions for the *in vitro* phagocytosis of heat killed *Saccharomyces cerevisiae* by human polymorphonuclear granulocytes in suspension, the characteristics of the interaction were studied with the aid of a Coulter Counter ZF and a pulse-height analyzer Channelyzer C-1000.

MATERIALS AND METHODS

Isolation of PMN cells. Human polymorphonuclear granulocytes (= PMN cells) were isolated from peripheral venous citrate blood on a density gradient (density 1 07) of sodium metrizoate (Nyegaard & Co AS Oslo Norway) and Macrodex® (Pharmacia, Uppsala, Sweden) according to *Bøyum* (1). In brief 10 ml of citrate blood was layered carefully on a mixture of 10 ml sodium metrizoate and 20 ml Macrodex® and after 45 min the leukocyte rich plasma was withdrawn with a Pasteur pipette. The plasma was then suspended in phosphate buffered saline (PBS pH = 7.3) and centrifuged at 1250 rev/min (= 200 *g*) for 15 min in a Sorvall SS-34 rotor (Ivan Sorvall, Newton, Connecticut 06470 U.S.A.).

6 ml of distilled water was added to the pellet for 25 s in order to lyse contaminating red cells. The reaction was stopped by adding 2 ml 3 4 per cent (w/v) PBS.

The cells were washed twice in Ca^{2+} free Krebs-Ringer phosphate buffer with 10mM glucose (KRG). More than 80 per cent of the cells were PMN cells.

Phagocytic prey. Baker's yeast, *Saccharomyces cerevisiae* (Jästbolaget AB Rotebro Sweden) was suspended in saline (0 15 M NaCl) heat killed at 100 C for 30 min and filtered through gauze. It was then washed twice in PBS and resuspended to 10^9 cells per ml. The cells were counted in a Bürker chamber and a Coulter Counter ZF with the apparatus set at A = 0.354 and I = 4. A threshold of T = 10 was used (8) (Coulter Electronics Ltd., High Street, South Dunstable, Beds England).

The same lot of yeast cells was used throughout the investigation. They were stored in aliquots at −20 C and washed twice in KRG before use.

Phagocytic system. The phagocytosis of yeast by the PMN cells was studied *in vitro* with both leucocytes and yeast cells in suspension (in KRG). The reaction system contained 5 per cent (v/v) homologous serum.

The PMN cells were preincubated at 37°C with serum, KRG and metabolic inhibitors in siliconized Erlenmeyer flasks, which were placed on a rotary shaker. After 20 min of incubation the yeast cells were added.

The influence of two metabolic inhibitors on the phagocytic process was studied. These are iodoacetamide (Dr T Schuchart, München, West Germany) and cytochalasin B (cyt B EGA-Chemie KG 7924 Steinheim bei Heidenheim, Brenz, West Germany). Analytical grade chemicals were used. Cyt B was dissolved to 250 µg/ml in 4 per cent (v/v) dimethylsulfoxide (DMSO).

Samples of 0.5 ml were withdrawn at 0, 15, 30 and 60 min after the addition of yeast cells and put in a beaker with 39.5 ml PBS for counting in the Coulter Counter. No further dilution was necessary for the counting of *S cerevisiae* and PMN cells.

The phagocytic process was also followed in the phase-contrast microscope.

Counting and sizing yeast and PMN cells. For counting *S cerevisiae* the Coulter Counter ZF was set as follows, A = 0.354 I = 4 and the number of particles counted at T (threshold) = 100 subtracted from those at T = 10 (8). For PMN cells, A = 1 I = 16 and the T-interval between 30 and 100 were normally used (see Fig. 2). On the Channelyzer the Base Channel Threshold (BCT) was = 4 the window width = 100 the counting time = 13 s and the integration range 10–99 and = 30–99 for yeast and PMN cells, respectively. With these settings more than 95 per cent of the populations were counted between the lower and upper threshold (T). A 100 µm capillary and a sampling volume of 0 5 ml were used.

RESULTS

Characteristics of the Phagocytic Process as Visualized by the Coulter Counter

Pulse height distribution of Saccharomyces cerevisiae. The bimodal pulse height (≈ volume) distribution of *Saccharomyces cerevisiae* suspended in PBS is shown in Fig 1. In the phagocytic system this distribution was

reproduced on a background of small particles. No overlapping with PMN cells was observed (Fig. 1). The figure also shows that the number of yeast cells decreased with time. Inspection in the phase-contrast microscope confirmed that an increasing number of yeast cells adhered to or were ingested by granulocytes.

No aggregation of the yeast cells was observed, i.e. no formation of particles with a pulse-height at least twice that of single cells. This was confirmed by means of the microscopical examination.

Pulse-height distribution of the granulocytes. After preparation of the PMN cell suspension, a small number of erythrocytes remained intact, as shown in Fig. 2. These constitute the minor peak centred at threshold value of about 10, when the PMN cells were suspended in PBS as well as in the phagocytic system. The PMN cells form the larger peak at a threshold of around 25. The curve nearest the axis shows that the background from yeast cells in the threshold interval T = 15–100 is small. Furthermore Fig. 2 shows two characteristic changes in the PMN cell distribution during phagocytosis. After 30 min of phagocytosis the distribution was moved slightly towards a higher threshold and the number of pulses diminished as larger particles were formed. The latter tendency was enhanced by a larger number of yeast

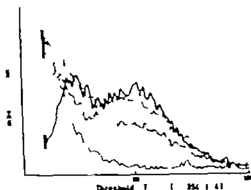


Fig. 1. Pulse-height distribution of *S. cerevisiae* (5×10^6/ml) in PBS, pH = 7.3 (——) in the reaction system with 10^6/ml PMN cells at 0 min ( ) and at 30 min (------). Background without *S. cerevisiae* (– –).

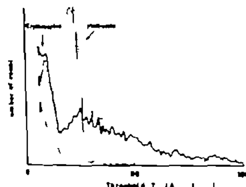


Fig. 2. Pulse-height distribution of PMN cells (10^6/ml) after 30 min in the reaction medium, with no *S. cerevisiae* (– –) with 1.5×10^6/ml ( ) and with 9×10^6/ml (——). Background without PMN cells (5×10^6/ml *S. cerevisiae* (------))

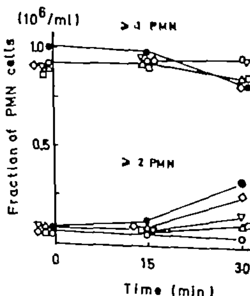


Fig. 3. Fraction of PMN cells (ordinate ≥1 PMN cell (upper graphs) and ≥ 2 PMN cells (lower graphs) at different times of reaction (abscissa) after reaction with different concentrations of *S. cerevisiae* per ml, ○ = 0, △ = 1×10^6, □ = 1.5×10^6, ▽ = 2×10^6, ◇ = 5×10^6, ● = 10×10^6. Initial concentration of PMN cells 1×10^6/ml.

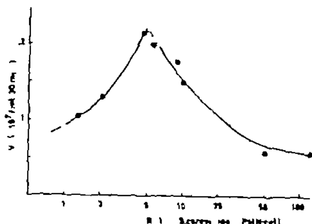


Fig 4 Phagocytic velocity v (= decrease of number of *S cerevisiae* per 10^6 PMN cells/ml and 30 min ordinate) at different ratios between the concentrations of *S cerevisiae* and PMN cells (abscissa)

cells (*S cerevisiae* PMN = 9 1) The tendency to aggregation, which was not observed in the control without yeast cells after 30 min could either be seen as a decreased number of particles ≥ 1 PMN cell or as an increased number of particles ≥ 2 PMN cells. The increased aggregation when more yeast cells were present could also be demonstrated quantitatively (Fig 3)

Reaction Kinetics

Phagocytic velocity The phagocytic velocity i.e the uptake of prey by the phagocyte during a certain time interval, is of fundamental importance for characterization of the phagocytic process (13)

In Fig 4 the decrease in number of yeast cells after 30 min is plotted as a function of the initial ratio between the concentration of yeast and PMN cells.

The graph shows clearly that the phagocytic velocity increased up to a yeast PMN ratio of around 5 whereas it was decreased by larger amounts of yeast cells.

Reaction order and influence of inhibitors of granulocyte metabolism In Fig 5 a, the fraction of non phagocytosed yeast cells, and in Fig 5 b the fraction of PMN cells ≥1 PMN cell, were plotted against time in the control and systems with 1 and 10 μg/ml cyt B

The curves are linear thus indicating that the reactions are of the first-order The interaction between yeast and PMN cells was gradually inhibited by increasing amounts of cyt B

Evaluation of Model for the Phagocytic Process

The uptake of a particle by a granulocyte has been considered as a two-step process, consisting firstly of an adhesion stage which is reversible and energy-independant, and secondly of an ingestion stage which is irreversible and energy-consuming.

Fig 6 and 7 show the influence of 2 mM iodoacetamide on the phagocytic velocity of *Saccharomyces cerevisiae* in linear (Fig 6) and reciprocal (= Lineweaver Burk (7) Fig 7) representation The V_{max} values in Fig. 6 are

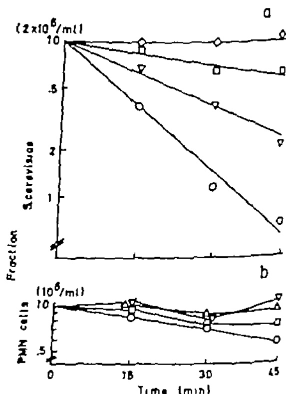


Fig 5 (a) Fraction of *S cerevisiae* and *(b)* PMN cells at different times of reaction in systems with cyt B ○ = S.c. + PMN ▽ = S.c. + PMN + 1 μg/ml cyt B □ = S c + PMN + 10 μg/ml cyt B, ◇ = S c. and Δ = PMN Initial concentration of *S cerevisiae* 2 × 10^6/ml and of PMN cells 1 × 10^6/ml.

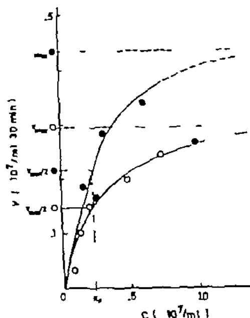


Fig. 6. Phagocytic velocity (= decrease of number of *S. cerevisiae* per 10⁶/ml PMN cells and 30 min) for different concentrations of *S. cerevisiae* in systems with iodoacetamide (linear representation) ● = no inhibitor ○ = 2 mM iodoacetamide

taken from Fig. 7 at the interception with the ordinate. The filled ring next to the ordinate in Fig. 7 was not taken into account, since judging from Fig. 6 this value does not obey first-order kinetics, as discussed earlier (Fig. 4).

DISCUSSION

Two principal ways may be used for *in vitro* modelling of the phagocytic process, i.e. with the phagocytes attached to a surface, or free in suspension. The relevance of the model chosen depends on the *in vivo* localization and mode of operation of the phagocyte. With the granulocytes, both models are relevant.

Many systems are indirect measurements of the phagocytic activity (20), e.g. O_2 consumption (18), myeloperoxidase mediated iodine incorporation (14), activation of the HMP-shunt (24) or the addition of radioactively labelled or coloured particles (19).

A more direct method is to follow the phagocytic process in the microscope either directly or cinematographically. However, this approach is cumbersome and restricts the analysis to a limited number of cells.

Furthermore, in the methods using radioactive or coloured particles, free and bound particles must be separated from each other.

With electronic particle counters such as the Coulter Counter and the Celloscope, particles which differ from one another by their volume can be separated electronically simply by choosing an appropriate window around the middle of the pulse-height distribution. The results (Fig. 1–2) show that this is possible with human PMN cells and *Saccharomyces cerevisiae*. Baker's yeast was used as prey in these studies, since it is a rather big ellipsoid-shaped microorganism (axis 3–5 µm) which can be detected easily in the microscope and in the counter. The latter needs a particle diameter of about 0.5 µm or higher for counting. Bacteria can also be counted, though care must be taken to avoid electrical noise from the electrical network or mismatch of the temperature in the suspending medium and the capillary bathing solution.

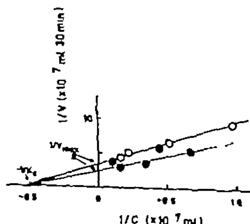


Fig. 7. Phagocytic velocity in systems with iodoacetamide (reciprocal, Lineweaver-Burk representation of Fig. 6) ● = no inhibitor ○ = 2 mM iodoacetamide

Attachment phase Engulfment phase



$$1/V = 1/k_3E_0 + (K_c/k_3E_0)1/C$$

$$K_c = (k_2 + k_3)/k_1$$

$$1/k_3E_0 = 1/V_{max}$$

Fig 8 Model of the phagocytic process. V is phagocytic velocity E_0 number of reaction loci k_1 k_2 and k_3 reaction constants (13)

Due to the interaction with the PMN cells, the number of yeast cells decreases. Besides the decrease in the number of free yeast cells, the modal or peak volume of the granulocytes is increased by "swelling" and aggregation (Fig 2 and 3) The "swelling" cannot be regarded simply as addition of yeast, since osmotic changes due to the phagocytic process cannot be excluded An increase of the functional resistivity of the particle would yield a similar effect (8) Such "swelling" has also been observed in a dose response way after the interaction of PMN cells with lipid vesicles (to be published) The disappearance of yeast cells could be inhibited by increasing concentrations of cyt B (5 and 10 μg/ml Fig 5 a) This substance blocks the glycolysis and the contraction of microfilaments (10 25) Consequently the decrease of yeast cells is assumed to be due to phagocytosis by the PMN cells This was confirmed by microscopical examination.

For a proper analysis of the phagocytic process, it is necessary to know the kinetics of the reaction (13) Furthermore, Fig 5 a indicates that reaction is of the first-order up to 45 min This is probably due to the low ratio (2 1) between the number of yeast and PMN cells. This assumption is supported by Fig 4 which shows that the ratio between the number of yeast and PMN cells must be below 5 1 without a reduction of the phagocytic velocity This implies that a zero-order reaction, i e. with an excess of the prey is not achievable Several mechanisms for the inhibition of the interaction at larger relative concentrations of the prey can be proposed, such as exhaust of serum factors (no phagocytosis was observed in a system without serum unpublished observation) release of toxic substances from the yeast, lack of energy in the granulocytes, alteration of the cell membrane on the granulocyte resting phase in the uptake or competition between the yeast particles (5)

Several attempts have been made to evaluate the phagocytic process by methods developed for enzymatic reactions, i.e with the PMN cells as the enzyme and yeast as the substrate (3 12, 13) The atachment phase is assumed to be reversible and characterized by the reaction constants k_1 and k_2 and the ingestion phase as irreversible and characterized by the constant k_3 (Fig 8) E_0 is the number of reaction loci and $1/K_c$ the affinity constant. See ref 13) It is notable that the iodoacetamide apparently does not affect $1/K_c$ i e. the attachment, whereas V_{max} i.e. k_3 is decreased (Fig 6 and 7) This is to be expected, since the ingestion phase is energy requiring and iodoacetamide inhibits the glycolysis (23) These figures again show clearly that care must be taken as regards the relative number of granulocytes and prey so that a relative decrease of the phagocytic velocity is avoided and consequently the same order of kinetics is maintained in the variation of the concentration of the prey

To summarize, we think particle counters such as the Coulter Counter with a pulse-analyzer might be a good complement to other methods for the assessment of phagocytosis. These permit the study of a large number of cells and several parameters, such as aggregation and volume changes, can be investigated. Different populations in the same suspension can be separated electronically and no labelling of the prey is required.

Furthermore, the results which are collected momentarily in the pulse-height analyzer can be stored, e.g. on tape, for further handling in a computer

The skilled technical assistance of Mrs. *Ellinor Granström* is gratefully acknowledged. This research was supported by the grant B76-16X-02183-10 from the *Swedish Medical Research Council.*

REFERENCES

1. *Böyum A.* Isolation of leukocytes from human blood. Further observations. Methylcellulose, dextran and Ficoll as erythrocyte aggregating agents. Scand. J. Clin. Lab. Invest. 21 Suppl. 97 31 50 1968.
2. *England J M & Down M C* Red-cell-volume distribution curves and the measurement of anisocytosis. Lancet April 20 701–703, 1974
3. *Gosselin, R. E.* The uptake of radiocolloids by macrophages in vitro. A kinetic analysis with radioactive colloidal gold. J. Gen. Physiol. 39 625–649 1956
4. *Grover N. B., Naaman J. Ben-Sasson S. & Doljanski F.* Electrical sizing of particles in suspensions. I. Theory. Biophys. J. 10 1398–1414 1969
5. *Hanks, J. H.* Quantitative aspects of phagocytosis as influenced by the number of bacteria and leukocytes. J. Immunol. 38 159–176, 1959
6. *Kubitschek H. E.* Counting and sizing microorganisms with the Coulter Counter. I. *Norris J. R. & Ribbons D. W.* (Eds.) Methods in Microbiology Vol. 1. Academic Press, New York, 1969 p. 593–610
7. *Lineweaver H. & Burk D.* The determination of enzyme dissociation constants. J. Am. Chem. Soc. 56 658, 1934
8. *Magnusson K.-E. & Edebo L.* Estimation of the disruption in freeze-pressed *Saccharomyces cerevisiae* by an electronic particle counter. Biotechnol. Bioeng. 16 1273–1282, 1974
9. *Magnusson K.-E. & Edebo L.* Cell resistivity measured by Coulter Counter. Poster 197. 5th Int. Biophysics Congress, Copenhagen, 1975.
10. *Malawista, S. E. Gee J. B. L. & Bensch K. G.* Cytochalasin B reversibly inhibits phagocytosis. Functional, metabolic, and ultrastructural effects on human blood leukocytes and rabbit alveolar macrophages. Yale J. Biol. Med. 44 286–300, 1971
11. *Mel H. C. & Yee J. P.* Erythrocyte size and deformability studies by resistive pulse spectroscopy. Blood Cells 1 391 599 1975.
12. *Mizuochi, T. Nishimura, Y., Sakai A. Takenaka O. & Ueda Y.* Kinetic study on phagocytosis of bovine leucocytes measured by oxygen uptake. FEBS Letters 51: 174–176, 1975.
13. *Normann, S. J.* Kinetics of phagocytosis. II. Analysis of in vivo clearance with demonstration of competitive inhibition between similar and dissimilar foreign particles. Lab. Invest. 31 161 169 1974
14. *Olsson I. Olofsson T. & Odeberg, H.* Myeloperoxidase-mediated iodination in granulocytes. Scand. J. Haematol. 9 482–491 1972.
15. *O'Neill, C. H. & Burnett M. E.* Inhibition of spontaneous aggregation by wheat germ agglutinin in suspensions of BALB/C-3T3 and BHK21 tissue culture fibroblasts. Exp. Cell Res. 83 247–254 1974
16. *Ottewill R. H. & Walker T.* Influence of particle size on the stability of polystyrene latices with an adsorbed layer of nonionic surface active agent. J. Chem. Soc. Faraday Trans. I 70 917–926, 1974
17. *Riemann F. Zimmermann J. & Pilwat G.* Release and uptake of haemoglobin and ions in red blood cells induced by dielectric breakdown. Biochim. Biophys. Acta 394 449–462, 1975
18. *Sbarra A. J. & Karnovsky M. L.* The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leucocytes. J. Biol. Chem. 234 1355–1362, 1959
19. *Stossel, T. P., Mason R. J. Hartwig, J. & Vaughan M.* Quantitative studies of phagocytosis by polymorphonuclear leukocytes: use of emulsions to measure the initial rate of phagocytosis. J. Clin. Invest. 51 615–624 1972.
20. *Stossel T. P.* Phagocytosis I III. N. Engl. J. Med. 290 717–723 774–780 833–839 1974
21. *Stossel, T. P.* Recognition and ingestion. Semin. Hematol. 12 83–116, 1975
22. *Talstad I. gebiegt.* The relationship between phagocytosis of polystyrene latex particles by polymorphonuclear leukocytes (PML) and aggregation of PML. Scand. J. Haematol. 9 516–523 1972.
23. *White A. Handler P. & Smith, E. L.* Principles of Biochemistry. McGraw-Hill, New York etc. Kōgakusha Company Ltd., 1968 p. 392 394
24. *Zatti M. Rossi, F. & Patriarca, P.* The H_2O-production by polymorphonuclear leukocytes during phagocytosis. Experientia 24 669–670 1968.
25. *Zigmond S. H. & Hirsch J. G.:* Effects of cytochalasin B on polymorphonuclear leucocyte locomotion, phagocytosis and glycolysis. Exp. Cell Res. 73 383–393 1972.

Acta path. microbiol scand Sect. C 85 222-224 1977

BRIEF REPORT

THE FINDING THAT SECRETORY COMPONENT IS NOT ASSOCIATED WITH γ-GLUTAMYLTRANSPEPTIDASE ACTIVITY

E. Lindh & I. Björk

Department of Medical and Physiological Chemistry
Royal Veterinary College The Biomedical Center
Uppsala, Sweden

Lindh E. & Björk, I., The finding that secretory component is not associated with γ-glutamyltranspeptidase activity Acta path. microbiol. scand. Sect. C, 85 222 224 1977

The γ-glutamyltranspeptidase activity of human milk was concentrated by ammonium sulphate precipitation On gel chromatography of the dissolved precipitate, the activity was eluted in the high-molecular weight fraction containing secretory IgA, while no activity appeared in the eluate at the position of free secretory component. Various antisera were added to portions of the pool of active fractions. No change of γ-glutamyltranspeptidase activity was obtained with antisera against either IgA secretory IgA or secretory component, while a large reduction of activity was seen with anti-human colostrum. Finally purified free secretory component, secretory IgA and *in vitro* complexes between secretory component and IgA dimers were shown to be inactive in the γ-glutamyltranspeptidase assay both in the absence and presence of zinc ions Thus secretory component either when free or bound to IgA does not exhibit γ-glutamyltranspeptidase activity and therefore cannot function as such an enzyme in the transport of IgA across mucous membranes, as has been suggested previously

Key words Secretory IgA secretory component γ-glutamyltranspeptidase.

Ingemar Björk, Department of Medical and Physiological Chemistry Royal Veterinary College Biomedicum, Box 575 S-751 23 Uppsala, Sweden

Received 8 iii 77 Accepted ix 77

Secretory immunoglobulin A the main antibody of human secretions, consists of two monomeric IgA units and two additional polypeptide chains viz. J-chain and secretory component (17 9) The functions of the two latter chains remain uncertain although the evidence generally suggests that J chain is of importance in the assembly of monomeric immunoglobulin units to polymers (9 14 10) and that secretory component may serve as a receptor for IgA, directing its transport through the epithelial cells of the mucosal membrane (15,5-7) Recently it was proposed that secretory component may be involved in this transport process by functioning as an enzyme with γ-glutamyltranspeptidase activity (2,3) It has been suggested previously that such enzymes participate in the transport of amino acids and peptides across cell membranes (16) However the experiments presented in this report demonstrate that secretory component both when free and when bound to IgA does not exhibit γ-glutamyltranspeptidase activity and that therefore this attractive hypothesis must unfortunately be incorrect.

Materials and Methods

Human secretory IgA secretory component, IgA dimers and *in vitro* complexes between IgA dimers and secretory component were prepared as described previously (4 12)

Human defatted and decaseinated milk was precipitated with ammonium sulphate at successively increasing saturation levels of 40 60 and 80 per cent The dissolved precipitates and the supernatant were

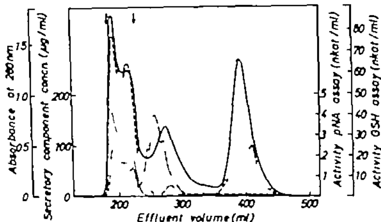


Fig 1 Gel chromatography on Sephadex G-200 of the protein fraction of human milk which precipitated when the ammonium sulphate saturation was raised from 40 to 60 per cent. About 160 mg of protein (based on specific absorption coefficient at 280 nm of 10) was applied to a 2.5×90 cm column in 0.05 M Tris buffer pH 8.0, containing 0.075 M sodium chloride. The elution rate was 12.5 ml/h and 3-ml fractions were collected.
—— absorbance at 280 nm enzyme activity in the γ-glutamyl-p-nitroanilide (pNA assay)
– – – enzyme activity in the glutathione (GSH) assay
– – – concentration of secretory component
The arrow indicate those fractions which were pooled and analysed further

dialyzed extensively against 0.05 M Tris buffer pH 8.0, containing 0.075 M sodium chloride and were then assayed for γ-glutamyltranspeptidase activity.

Two methods were used for determination of γ-glutamyltranspeptidase activity. One was based on the transfer of the γ-glutamyl group from γ-glutamyl-p-nitroanilide to glycylglycine with the concomitant release of p-nitroaniline. This reaction was observed spectrophotometrically at 410 nm. Details of the procedure have been published by *Tate & Meister* (16). The other method measured the transfer of the γ-glutamyl group from glutathione to glycylglycine, under the conditions used by *Leibach & Binkley* (11). The disappearance of glutathione was measured as described by *Ball* (1). The enzyme activity obtained with both methods was expressed in nanokatals (nkat) i.e. the amount of activity corresponding to the conversion of 1 nmol om substrate per second.

Determination of the concentration of secretory component in column eluates was performed by single radial immunodiffusion, as described by *Mancini et al.* (13). Both free and bound secretory component reacted with the antiserum used.

All antisera used in this investigation were purchased from Dakopatts A-S (Hellerup, Denmark).

Results and Discussion

The γ-glutamyltranspeptidase activity in human milk was found to be concentrated in the protein fraction which precipitated when the ammonium sulphate saturation was increased from 40 to 60 per cent. About 75 per cent of the total activity measured by both assay methods, was recovered in this precipitate. A portion of the protein fraction was applied to a Sephadex G-200 gel chromatography column, and the effluent was analysed for protein by ultraviolet absorbance, for γ-glutamyltranspeptidase activity by both assay methods, and for secretory component by single radial immunodiffusion (Fig. 1). The chromatograms show that most of the enzyme activity appeared in the first, bimodal, protein peak (which contains secretory component associated with IgA, i.e. secretory IgA) and that no activity was seen in the second peak at the elution position of free secretory component. Two peaks with activity in the glutathione assay but without activity in the γ-glutamyl-p-nitroanilide assay were found later in the chromatogram. These peaks may reflect the presence of enzymes capable of oxidizing or modifying glutathione in some other manner than transfer of its γ-glutamyl group to an acceptor.

The experiment described thus suggests that free secretory component is devoid of γ-glutamyltranspeptidase activity but does not exclude the possibility that secretory component may exhibit such activity when bound to IgA in the form of secretory IgA. Further experiments where undertaken to test this possibility. The gel chromatographic fractions containing the γ-glutamyltranspeptidase

activity were pooled as indicated in Fig 1 and the effect of the addition of various antisera to portions of this pool was tested. Table 1 shows that no change of γ-glutamyltranspeptidase activity (measured by both assay methods) was obtained with antisera against either IgG IgA secretory IgA or secretory component, and that the only reduction of activity was shown by the positive control i.e. an antiserum against whole human colostrum. Anti IgG was included in the test only to reveal a possible effect of the antisera on the enzyme assays. Other amounts of antisera than those presented in the table were also used with essentially the same result. These experiments thus show that secretory component whether free or bound, does not show γ-glutamyltranspeptidase activity and that therefore this activity found in human milk must reside in some protein other than secretory IgA or secretory component.

TABLE 1 *Effect on γ-Glutamyltranspeptidase Activity of the Addition of various Antisera to a Pool of Active Fractions from the Gel Chromatography of Fig 1*

Antiserum	Enzyme activity (nkat/ml) γ-glutamyl-p-nitroanilide assay	glutathione assay
Buffer	1 35	30 0
Anti-IgG	1 34	32.4
Anti-IgA (heavy chain)	1 25	31 6
Anti-secretory IgA	1 34	34 5
Anti secretory component	1 38	33.5
Anti human colostrum	0 15	12.8

A volume of 450 μl of the pool from the gel chromatogram (protein concentration about 0 7 mg/ml) was mixed with 150 μl of antiserum and the mixture was incubated at 37 C for 1 h. After centrifugation appropriate portions of the incubation mixture were taken for analysis of enzyme activity

Further verification of this conclusion was obtained when purified proteins were tested for transpeptidase activity in the γ-glutamyl-p-nitroanilide assay These analyses were performed both in the absence and presence of 1 mM $ZnCl_2$, since it has been suggested that exposure to phosphate buffers (which were used in our preparatory procedures) lead to the dissociation of essential zinc ions from the enzyme (3) These experiments showed that both purified free secretory component and purified secretory IgA had no activity whatsoever regardless of whether or not zinc was included in the assay mixtures. Moreover, several *in vitro* complexes between secretory component and IgA dimers (12) were also tested and found to be inactive In further support of the contention that no induction of γ-glutamyltranspeptidase activity occurs when secretory component binds to IgA.

In conclusion, the different experiments presented here show that secretory component, whether free or bound to IgA, does not exhibit γ-glutamyltranspeptidase activity Thus, the suggested participation of secretory component in the transport of IgA across mucous membranes cannot be explained on the basis of such enzymatic activity The mechanism of this transport process therefore still remains illusive.

Finally it can be added that during the preparation of this report, a paper by *Brandtzaeg* & *Wiswner* appeared (8) showing that secretory component was devoid of γ-glutamyltranspeptidase activity in a γ-glutamyl-p-nitroanilide assay system

This investigation was supported by a research grant from the *Swedish Medical Research Council* (Project No. 04212) We are indebted to Miss *K Larsson* for excellent technical assistance

References 1 *Ball C R.* Biochem. Pharmacol *15* 809–816 1966.—2 *Binkley F* & *Wiesemann, M L.* Life Sci. *17* 1359–1361 1975—3 *Binkley, F Wiesemann M L. Hand W L Johnson J D King V L. Groth D P* & *Powell R.* W Fed. Proc. *34* 513 1975—4 *Björk I* & *Lindh, E* Eur J Biochem. *45* 135–145 1974—5 *Brandtzaeg P* Nature New Biol. *243* 142–143 1973—6 *Brandtzaeg P* Immunology *26* 1101 1114, 1973—7 *Brandtzaeg P* J Immunol. *112* 1553–1558 1974—8 *Brandtzaeg P* & *Wisner A.* Immunochemistry *13* 951—954 1976.—9 *Halpern, M S* & *Koshland M E* Nature (Lond) *228* 1276–1278 1970—10 *Halpern M S* & *Koshland, M E* J Immunol. *111* 1653–1660 1973.—11 *Leibach F H* & *Binkley F* Arch. Biochem. Biophys. *127* 292–301 1968.—12 *Lindh E* & *Björk I* Eur J Biochem. *45* 261–268, 1974—13. *Mercin G Carbonara A-O* & *Heremans J F* Immunochemistry 2 235–254 1965—14 *Morrison S L.* & *Koshland M E* Proc. Natl Acad. Sci. U.S.A. *69* 124–128 1972—15 *South M A., Cooper M D Wollheim F A Hong, R.* & *Good R A* J Exp. Med *123* 615–627 1966.—16. *Tate S S* & *Meister A* J Biol. Chem. *249* 7593–7602, 1974—17 *Tomasi T B Tan E Solomon A* & *Prendergast R A* J Exp Med. *121* 101–124 1965

Acta path. microbiol. scand. Sect. C, 85: 225-232, 1977

BOVINE MYELIN BASIC PROTEIN IMMUNOCHEMICAL SPECIFICITY EXAMINED BY THE MACROPHAGE MIGRATION INHIBITION TEST

HÅKAN BERGSTRAND

Tornblad Institute, University of Lund, Sweden and AB Draco, Lund, Sweden

Bergstrand, H. Bovine myelin basic protein. Immunochemical specificity examined by the macrophage migration inhibition test. Acta path. microbiol. scand. Sect. C, 85: 225-231 1977

Guinea pigs were injected with saline or with one of the following antigens incorporated into Freund' complete adjuvant: Bovine myelin basic protein (MBP) lysozyme, carboxymethylated lysozyme, or crude commercial calf thymus histone preparation. Examination of the migration of peritoneal cells from these animals in the presence (50 µg/ml) or the absence of the antigens revealed, at most, a slight one-way form of cross-reactivity between MBP on the one hand and the histone preparation—and possibly also lysozyme—on the other. This was observed only with cells from animals injected with the two last-mentioned antigens. Cells from animals sensitized with either histone or lysozyme mixed with poly AU instead of Freund' complete adjuvant were also slightly inhibited in their migration by the bovine MBP. Cells from animals injected with complete Freund adjuvant alone or with the purified protein derivative of tuberculin (PPD) in poly AU did not react to MBP. Thus, in contrast to the high degree of internal cross-reactivity shown previously MBP shows low degree of cross-reactivity with either ordered or unordered forms of some unrelated basic proteins.

Key words: Bovine myelin basic protein; immunochemical specificity; migration inhibition.

H. Bergstrand, AB Draco, Fack, S-221 01 Lund, Sweden.

Received 1.ii.77 Accepted 1.iii.77

Myelin basic protein (MBP—also called encephalitogenic protein) is a constituent of central nervous system myelin. It has been studied extensively during the past decade because it induces an experimental disease—allergic encephalomyelitis (EAE)—thought to be primarily due to cell-mediated autoimmunity against the protein (for recent reviews, see *Kies* 1973 and *Rauch & Einstein* 1974). These studies were aimed initially at defining the chemical basis for disease induction and its relation to the cell-mediated immune response to MBP. However they have also provided considerable knowledge concerning the general immunochemical char

Abbreviations

CM-lysozyme = Carboxymethylated lysozyme.
FCA = Freund' complete adjuvant.
FIA = Freund's incomplete adjuvant.
MBP = myelin basic protein.
poly A = polyadenylic acid.
poly U = polyuridylic acid.
PPD = purified protein derivative (of mycobacterial origin).

acteristics of the protein (reviewed by *Kies* 1973 1975 *Rauch & Einstein* 1974, *Bergstrand* 1977) The primary sequences of different MBPs show an appreciable degree of homology (see *Dunkley & Carnegie* 1974) However the degree and specificity of an immune response towards a specified part of MBP seem to depend on (i) the animal species employed for evaluation and (ii) the origin of the antigen. This can be exemplified by the species specificity encountered during studies of various encephalitogenic determinants (for review, see *Bergstrand* 1977) Furthermore immunological cross reactivity on the humoral level has not been established conclusively between MBP and any other protein *Kornguth et al* (1972) and *Whittingham et al* (1972) claimed partial immunochemical identity between MBP and histones, but the findings have not been confirmed (*Schmid et al* 1974 *Bustin et al.* 1975 *Whitaker* 1975)

Some protein antigens cross-react at the cellular level but not at the humoral level of the immune response (for references see *Yamashita et al* 1975) In agreement with the above mentioned observations, *Bustin et al* (1975) reported that bovine MBP and histone F 2A 1 cross-react in tests for delayed skin reactivity and inhibition of antigen induced lymphoid cell transformation, but not when examined at the humoral level. Similarly the finding that peptides originating in the three main parts of MBP show a substantial degree of cross-reactivity when examined with techniques primarily thought to reflect T-cell responses (*Bergstrand* 1973 *Bergstrand & Kallén* 1973 a 1975 *Hashim & Schilling* 1973 *Hashim et al* 1973 *Hashim & Sharpe* 1974) apparently has no correspondence at the humoral level (*Driscoll et al.* 1974)

Several authors suggested the existence of some kind of cellular cross-reactivity between MBP and mycobacterial antigens (? PPD) (see, e.g *Hughes et al* 1970 *Bergstrand & Kallén* 1973 b *McDermott et al* 1974 *Vandenbark et al* 1975) *McDermott et al* (1974) used cellular affinity chromatography to deplete human lymphocyte populations of cells specifically reactive to MBP or PPD Subsequent measurement of the reactivity of these cells in the macrophage electrophoretic mobility test indicated that removal of cells sensitive to MBP was accompanied by a reduction of cells sensitive to PPD, and *vice versa* An analogous approach failed to show cross reactivity between MBP and histone, as claimed previously by *Johns et al.* (1973) *Vandenbark et al.* (1975) demonstrated a delayed dermal reactivity to MBP in guinea pigs heavily immunized with mycobacterial antigens. It was claimed that animals injected with MBP in incomplete adjuvant showed dermal hypersensitivity to mycobacterial antigens. On the other hand, *Day & Pitts* (1974) who observed the induction of anti MBP antibody formation in Lewis rats sensitized to FCA only also made the important observation that these antibodies failed to cross-react with mycobacterial constituents in adsorption tests.

The present report deals with the immunochemical relationship as expressed in the guinea pig macrophage migration inhibition test between MBP a crude commercial histone preparation PPD, and hen egg white lysozyme a basic protein not hitherto implicated in any type of cross-reactivity with MBP (see *Teitelbaum et al* 1971 *Webb et al* 1973 *Bustin et al* 1975 *Whitaker* 1975)

MATERIAL AND METHODS

Animals and Sensitization

Outbred guinea pigs, weight about 200–300 g, obtained locally were sensitized by injecting 0.05 ml of an antigen-adjuvant emulsion into each of three footpads. The emulsions were either prepared with Freund's complete adjuvant (FCA) or with Freund's incomplete adjuvant (FIA) In the latter case stock solutions of antigen were mixed before emulsification with an equal volume of a solution of poly AU (prepared from poly A and poly U [Sigma Co] as described by *Paterson & Drobish* (1974)) Each animal received 37.5 µg antigen in FCA or the same amount together with 600 µg poly AU in FIA. One group received FCA emulsified with saline only

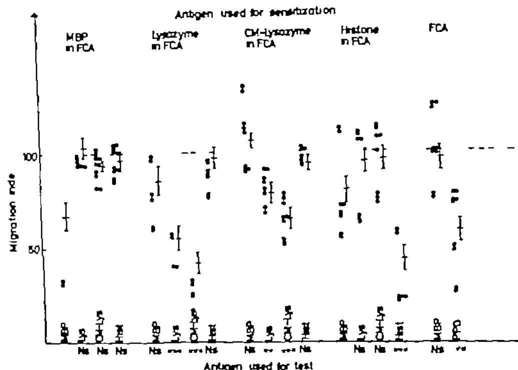


Fig 1 Migration indices recorded with cells from animals immunized with 37.5 μg of the indicated antigen in FCA. Cells from each group of animals were tested with the four antigens on the same occasion. Antigen concentration in migration chamber 50 μg/ml. The mean migration index for each antigen within each group is indicated. Vertical lines represent S.E.M. Probability levels for mean migration indices estimating 100 indicated at the bottom of the figure ns = non significant, *0.05 > p > 0.01 **0.01 > p > 0.001 ***0.001 > p. Mean migration index for cells from normal animals cultured with MBP (50 μg/ml) is 99.1

Antigens

The general procedure for preparing and characterizing the bovine MBP and the peptide derivatives of MBP used has been described previously (*Bergstrand* 1971 1973) Numbering of the amino acids was changed to conform to the revised primary sequence of bovine MBP (*Brostoff et al.* 1974) Hen egg white lysozyme and calf thymus histone were from Sigma Corp. Carboxymethylated lysozyme (CM-lysozyme) was prepared according to *Canfield & Anfinsen* (1963) On account of partial insolubility immunization with this antigen preparation was performed with suspension emulsified in FCA. PPD was obtained from Statens Seruminstitut, Copenhagen.

Migration Inhibition Technique

The technique used previously (*Bergstrand* 1973 *Bergstrand & Källén* 1972 1973 c) was modified slightly as follows. Paraffin oil induced peritoneal cells from actively sensitized animals were collected in about 100 ml cold medium 199 (SBL, Stockholm, Sweden) containing 2500 U of heparin (Vitrum, Sweden). The cells were washed three times (the last two were performed with heparin-free medium 199). Disposable micro pipettes (25 μl Drummond) were used as capillaries. Migration experiments were performed either in all glass chambers as described previously or in disposable plastic chambers (Kartell 357/4). For the plastic chambers, one capillary was placed in each chamber (containing approximately 0.3 ml medium). The basal culture medium consisted of 15 per cent heat inactivated normal guinea pig serum in 85 per cent medium 199 supplemented with antibiotics (25 U/ml benzyl penicillin and 25 μg/ml streptomycin sulphate) and 1 per cent 0.6 M $NaHCO_3$. Control cultures received 5 per cent saline, experimental cultures 5 per cent of the pertinent antigen (the final concentration mostly being 50 μg/ml). Four or more capillaries were set

acteristics of the protein (reviewed by *Kies* 1973, 1975 *Rauch & Einstein* 1974 *Bergstrand* 1977) The primary sequences of different MBPs show an appreciable degree of homology (see *Dunkley & Carnegie* 1974) However, the degree and specificity of an immune response towards a specified part of MBP seem to depend on (i) the animal species employed for evaluation and (ii) the origin of the antigen. This can be exemplified by the species specificity encountered during studies of various encephalitogenic determinants (for review see *Bergstrand* 1977) Furthermore, immunological cross-reactivity on the humoral level has not been established conclusively between MBP and any other protein *Kornguth et al* (1972) and *Whittingham et al* (1972) claimed partial immunochemical identity between MBP and histones, but the findings have not been confirmed (*Schmid et al* 1974 *Bustin et al* 1975 *Whitaker* 1975)

Some protein antigens cross-react at the cellular level but not at the humoral level of the immune response (for references see *Yamashita et al* 1975) In agreement with the above mentioned observations, *Bustin et al* (1975) reported that bovine MBP and histone F 2A 1 cross-react in tests for delayed skin reactivity and inhibition of antigen induced lymphoid cell transformation, but not when examined at the humoral level. Similarly the finding that peptides originating in the three main parts of MBP show a substantial degree of cross-reactivity when examined with techniques primarily thought to reflect T-cell responses (*Bergstrand* 1973 *Bergstrand & Kallén* 1973 a 1975 *Hashim & Schilling* 1973 *Hashim et al* 1973 *Hashim & Sharpe* 1974) apparently has no correspondence at the humoral level (*Driscoll et al.* 1974)

Several authors suggested the existence of some kind of cellular cross-reactivity between MBP and mycobacterial antigens (? PPD) (see, e.g. *Hughes et al* 1970 *Bergstrand & Kallén* 1973 b *McDermott et al.* 1974 *Vandenbark et al* 1975) *McDermott et al* (1974) used cellular affinity chromatography to deplete human lymphocyte populations of cells specifically reactive to MBP or PPD Subsequent measurement of the reactivity of these cells in the macrophage electrophoretic mobility test indicated that removal of cells sensitive to MBP was accompanied by a reduction of cells sensitive to PPD and *vice versa*. An analogous approach failed to show cross-reactivity between MBP and histone, as claimed previously by *Johns et al.* (1973) *Vandenbark et al.* (1975) demonstrated a delayed dermal reactivity to MBP in guinea pigs heavily immunized with mycobacterial antigens. It was claimed that animals injected with MBP in incomplete adjuvant showed dermal hypersensitivity to mycobacterial antigens. On the other hand, *Dau & Pitts* (1974) who observed the induction of anti-MBP antibody formation in Lewis rats sensitized to FCA only also made the important observation that these antibodies failed to cross-react with mycobacterial constituents in adsorption tests.

The present report deals with the immunochemical relationship as expressed in the guinea pig macrophage migration inhibition test between MBP a crude commercial histone preparation, PPD and hen egg white lysozyme, a basic protein not hitherto implicated in any type of cross reactivity with MBP (see *Teitelbaum et al.* 1971 *Webb et al* 1973 *Bustin et al* 1975 *Whitaker* 1975)

MATERIAL AND METHODS

Animals and Sensitization

Outbred guinea pigs, weight about 200–300 g, obtained locally were sensitized by injecting 0.05 ml of an antigen-adjuvant emulsion into each of three footpads. The emulsions were either prepared with Freund's *complete* adjuvant (FCA) or with Freund's incomplete adjuvant (FIA) In the latter case stock solutions of antigen were mixed before emulsification with an equal volume of a solution of poly AU (prepared from poly A and poly U [Sigma Co] as described by *Paterson & Drobish* (1974)) Each animal received 37.5 µg antigen in FCA or the same amount together with 600 µg poly AU in FIA. One group received FCA emulsified with saline only

TABLE 1 *Regression Coefficients ( ) Calculated from Data from Migration Experiment Shown in Fig. 1*

Immunizing antigen		Test antigen MBP	Lysozyme	CM-lysozyme	Histone	PPD
MBP	$r \pm s_r$ a)	0.67 ± 0.065	0.88 ± 0.039	0.94 ± 0.024	0.93 ± 0.035	ND
in FCA	n b)	13	13	13	13	
mean s_r = 0.0408	t = 1	8.07***	0.49	1.52	1.74	
Lysozyme	$r \pm s_r$	0.84 ± 0.055	0.72 ± 0.081	0.53 ± 0.058	0.92 ± 0.030	ND
in FCA	n	10	10	10	10	
mean s_r = 0.0508	t = 1	3.23	3.54 **	8.31***	1.63	
CM-lysozyme	$\pm s_r$	1.06 ± 0.043	0.82 ± 0.035	0.69 ± 0.057	0.95 ± 0.031	ND
in FCA	n	12	12	12	12	
mean s_r = 0.0464	t = 1	1.38	3.81**	6.78***	1.11	
Histone	$r \pm s_r$	0.77 ± 0.067	1.01 ± 0.032	0.97 ± 0.053	0.44 ± 0.061	ND
in FCA	n	12	12	12	12	
mean s_r = 0.0581	t = 1	3.90**	0.17	0.45	9.64**	
FCA	$\pm$	0.93 ± 0.055	ND	ND	ND	0.53 ± 0.068
	n	13				13
mean s_r = 0.0601	t = 1	1.14				7.83***

) Student t test performed with weighted mean s values within each group of animals.
b) *n* = *number* of experiments. Significance levels as in Fig. 1

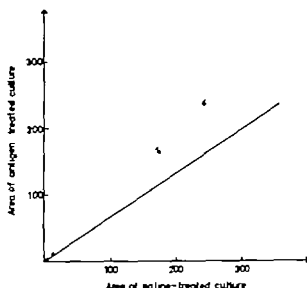


Fig 2 Migration areas recorded for cells from animals immunized with MBP in the presence of the immunizing antigen plotted against those recorded with the same cell population cultured with saline Regression line through origo showing best fit (least squares analysis) is drawn solid hatched line represents regression line for hypothetical unreaction antigen. Regression coefficient r = 0 67 ± 0 065 t_{n-1} = 5.03 n = 13 p<0 01

up with each additive Cultures were incubated for 18 hours at 37 C and traced on transparent paper at 20 × magnification. The migration area was measured by planimetry

RESULTS

Figure 1 shows the data from experiments with cells from animals sensitized to MBP lysozyme CM lysozyme or calf thymus histone in FCA, or to FCA alone. Besides the individual migration indices, the mean migration indices for each antigen with standard errors are given. The migration index is sometimes a rough estimate of the migration inhibition (*Bergstrand & Kallén* 1973 c) Therefore the primary data were also subjected to a simple regression analysis. For each group of animals, the migration areas of antigen treated cultures were plotted against the corresponding areas of saline-treated control cultures. Fig 2 illustrates this method and shows the results obtained with cells from animals sensitized to MBP tested with saline (x-axis) and MBP (y-axis) A regression line through the origo has been fitted to these data (a regression line not *a priori* fixed through origo does not improve the fit to the data) The regression coefficient r = 0 67 (which represents a mean migration index) deviates significantly from unity, which means that the antigen inhibits the cell migration. Table 1 gives the results obtained when the data from Fig 1 are thus treated. The regression coefficients of Table 1 and the mean migration indices of Fig 1 do not correspond exactly but the differences are slight. Table 1 indicates that 1) cells from animals sensitized to MBP in FCA show reactivity to the immunizing antigen only 2) cells from animals sensitized to lysozyme in FCA show clearcut reactivity to lysozyme and an even better reactivity to CM lysozyme and they possibly also react slightly to MBP 3) cells from animals sensitized to CM-lysozyme in FCA show reactivity to CM-lysozyme and to lysozyme, but apparently not to MBP 4) cells from animals sensitized to the histone preparation show reactivity to the immunizing antigen and to a lesser degree, to MBP and 5) cells from animals sensitized to FCA only do not show reactivity to MBP

Table 2 gives the results of analogous experiments with cells from animals sensitized to histone or to lysozyme in combination with poly AU and FIA. These experiments show 1) that sensitization to lysozyme also induces a slight degree of reactivity to MBP which apparently can be recalled by each of the three major peptide parts of MBP and also by the derivative that has been blocked at the tryptophan residue and 2) that sensitization to the crude histone preparation induces simultaneous sensitization to MBP in this case, reactivity of isolated peptide parts of MBP has not been ascertained.

We reported previously that cells from animals sensitized to FCA, but not normal cells, show enhanced migration in the presence of 50 µg/ml of peptide 1–42 of MBP Since then the data have been extended. The mean migration index recorded for the first mentioned cells in the presence of 50 µg/ml peptide 1–42 is MI = 108 17 ± 1 60 (111 d.f) p>0.001 for the same kind of cells in

…h Cells from Animals Sensitised with the Pertinent Antigen in Poly AU and FIA

…igen PPD	1–42	43–115 tyr	116–169 tyr	HNB-MBP
ND	0.92 ± 0.041 11 2.56*	0.92 ± 0.020 10 2.42*	0.93 ± 0.031 10 2.23	0.885 ± 0.037 10 3.51**
ND	0.99 ± 0.041 16 0.14	0.96 ± 0.046 16 0.79	0.91 ± 0.046 16 2.12	0.86 ± 0.053 15 2.21**
0.56 ± 0.103 8 7.93***	1.01 ± 0.02 8 0.18	ND	ND	ND

b) n = number of experiments. Significance levels as in Fig. 1

with PPD in poly AU and exposed cells from these animals to peptide 1–42. No effect on the migration of these cells was observed. These results lend no support to the possibility that PPD is a constituent of FCA cross-reacting with MBP.

In conclusion, the present results indicate that, as opposed to a high degree of internal cross-reactivity MBP shows a low degree of cross-reactivity with either ordered or unordered forms of some other basic proteins when examined in the guinea pig macrophage migration inhibition test.

The skilful technical assistance of Mrs. *Ingegerd Andersson* is gratefully acknowledged. The cost of the present examination was defrayed with grants from the *Swedish Medical Research Council*.

REFERENCES

Bergstrand, H. Isolation and partial characterization of some proteolytically and chemically derived fragments of bovine encephalitogenic protein. Eur. J. Biochem. *21*: 116–124, 1971.

Bergstrand, H. Localization of antigenic determinants on bovine encephalitogenic protein. Further studies with the macrophage migration inhibition assay in guinea pigs. Immunochemistry *10*: 611–620, 1973.

Bergstrand, H. Immunochemistry of Encephalitogenic Protein. In: *Atassi, M. Z.* (Ed.): Immunochemistry of Proteins. Vol. 1. Plenum Press, New York 1977, pp. 315–370.

Bergstrand, H. & Källén, B. Localization of antigenic determinants on bovine encephalitogenic protein. Studies in guinea pigs with macrophage migration inhibition. Cell. Immunol. *3*: 660–671, 1972.

Bergstrand, H. & Källén, B. Is there a cross-reactivity between different parts of the bovine encephalitogenic protein in the macrophage migration inhibition assay? Immunochemistry *10*: 471–476, 1973 a.

Bergstrand, H. & Källén, B.: Antigenic determinants in the N-terminal part of bovine encephalitogenic protein studied with the macrophage migration inhibition assay in guinea pigs. Evidence for an immunological reactivity of peptide 1–43 when tested on cells from animals injected with Freund' complete adjuvant. Immunochemistry *10*: 229–238, 1973 b.

Bergstrand, H. & Källén, B.: On the statistical evaluation of the macrophage migration inhibition assay. Scand. J. Immunol. *2*: 173–187, 1973 c.

Bergstrand, H. & Källén, B.: Further evidence of an extensive cross-reactivity between the three main parts of the bovine encephalitogenic protein in the lymphoid cell transformation test and the macrophage migration inhibition test. Acta path. microbiol. scand. Sect. C, *83*: 165–172, 1975.

Brostoff, S. W., Reuter, W., Hichens, M. & Eylar, E. H.: Specific cleavage of the A1 protein from myelin with cathepsin D. J. Biol. Chem. *249*: 559–567, 1974.

Bustin, M., Teitelbaum, D. & Webb, C.: Immunological comparison of basic encephalitogen and histone F2A1. Eur. J. Biochem. *53*: 615–621, 1975.

Canfield, R. E. & Anfinsen, C. B.: Chromatography of pepsin and chymotrypsin digests of egg white lysozyme on phosphocellulose. J. Biol. Chem. *238*: 2684–2690, 1963.

TABLE 2. *Regression Coefficients (r) Calculated from Migration Data Obtained*

Immunizing antigen		MBP	Lysozyme	Histone
Lysozyme with poly AU	r ± s a)	0 90 ± 0 035	0.37 ± 0 060	ND
	n b)	11	11	
	$t_{r=1}$	3 00*	13 07***	
Histone with poly AU	r ± s	0.89 ± 0 029	ND	0.68 ± 0.041
	n	16		16
	t = 1	2.52*		7.57***
PPD with poly AU	r ± s	1 11 ± 0 043	ND	ND
	n	8		
	t = 1	1 87		

a) Student's t tests performed with mean weighted s -values within each group of animals.

the presence of 25 μg/ml of peptide 1–42 it is 95.3 ± 4 68 (21 d f) p>0 05 and for normal cells or cells from animals sensitized with poly AU in FIA in the presence of 50 μg/ml of 1–42 it is MI = 101.97 ± 3 81 (31 d.f) p>0 05

As Table 2 shows, there is no indication that cells from animals sensitized to PPD in poly AU react with enhanced migration in the presence of peptide 1–42

DISCUSSION

The possible relationship between myelin basic protein and histones, natural and denatured forms of lysozyme and PPD was investigated as regards reactivity in the guinea pig peritoneal cell migration inhibition test The results indicate that there is only a faint degree of cross reactivity between MBP and the other antigens examined This is in contrast to the more extensive degree of cross-reactivity between different parts of MBP recorded previously In the present series of experiments, peritoneal cells from guinea pigs sensitized to a commercial preparation of histones or to native but not denaturated lysozyme tend to react to MBP in the migration inhibition test. However in the reciprocal experiments, cells from animals sensitized to MBP failed to react significantly to histone or lysozyme Immunochemical cross-reactivity between MBP and lysozyme has not been reported previously but *Bustin et al.* (1975) found that purified histone F2A1 cross-reacts with bovine MBP in delayed skin tests and with respect to its capacity to inhibit antigen induced lymphoid cell transformation tests in guinea pigs, in which case reciprocal cross-reactivity was observed. The crude nature of the histone preparation used in the present experiments might explain this discrepancy Thus the amount of the cross-reacting principle in the crude preparation might conceivably be sufficient for the induction of immunity at sensitization, but not for the expression of immunoreactivity to MBP in the *in vitro* test. Our finding that all three of the major parts of MBP seem to contribute to the cross-reactivity pattern is in agreement with previous findings showing an extensive degree of internal cross-reactivity within MBP

We reported previously (*Bergstrand & Källén 1973 b*) that cells from animals sensitized to FCA show enhanced migration in the presence of 50 μg/ml (approximately 10 μM) of peptide 1–42 of bovine MBP A similar observation was reported by *Lisak & Zweiman (1974)* One interpretation of this phenomenon might be the existence of immunochemical cross-reactivity between MBP (region 1–42) and some mycobacterial constituent We therefore immunized guinea pigs

Acta path. microbiol. scand. Sect. C, 85 233–238 1977

IN VITRO STUDIES ON NORMAL STIMULATED AND IMMUNOLOGICALLY ACTIVATED MOUSE MACROPHAGES

I Oxidation of 1 C Glucose by Macrophages in Monolayer Cultures

S Rhigaard, J Bennedsen and J M. Rhodes

Tuberculosis Department and WHO Collaborative Centre for Reference and Research on Escherichia, Statens Seruminstitut, Copenhagen, Denmark

Rhigaard, S., Bennedsen, J & Rhodes, J M. *In vitro* studies on normal, stimulated and immunologically activated mouse macrophages. I Oxidation of 1-^{14}C glucose by macrophages in monolayer cultures. Acta path. microbiol. scand. Sect. C, 85 233–238, 1977

Oxidation of 1 ^{14}C glucose of BCG/listeria activated, proteose-peptone stimulated and typhoid parathypoid vaccine (T.A.B.) stimulated peritoneal and spleen macrophages from C3H, CBA and C57Bl mice was determined at various times after treatment. The study showed no major differences between the oxidation of stimulated and activated macrophages, but a variation was seen in C3H mice in the kinetics of glucose oxidation of BCG/listeria activated and proteose-peptone stimulated peritoneal macrophages. It was also observed, in these mice that the response appeared and disappeared earlier in the spleen than in the peritoneal macrophages.

Key words Macrophages activation *in vitro* mouse glucose oxidation.

J Bennedsen, Tuberculosis Department, Statens Seruminstitut, Amager Boulevard 80, DK 2300 Copenhagen S, Denmark.

Received 3.I.77 Accepted 3.II.77

Activation of macrophages is considered to be brought about immunologically being mediated by factors liberated from T lymphocytes. This activation usually involves an increase in microbicidal activity (14) number of lysosomes (11) content of lysosomal enzymes (11) spreading of macrophages on glass (2) phagocytosis of particles (6, 10) and oxidation of glucose through the hexose-monophosphate shunt (6, 10)

However these changes in macrophage functions might also be caused by non-specific stimulation. For example, peritoneal macrophages can be stimulated *in vitro* by new born calf serum and show an increase in content of lysosomes, lysosomal enzymes and phagocytosis of *Toxoplasma gondii* and *Listeria monocytogenes* but they are rather ineffective in restricting intracellular growth of listeria (11) Elicitation of peritoneal macrophages with proteose-peptone yields macrophages, morphologically resembling activated cells (12) which show enhanced pinocytosis (5) and increased oxidation through the hexose monophosphate shunt (6) The question is whether activated and stimulated macrophages are essentially similar in many of their functional aspects.

It is, therefore of interest to ascertain, by *in vitro* tests, whether it is possible to distinguish between specifically activated macro-

Day E D & *Pitts O M* The antibody response to myelin basic protein (BP) in Lewis rats The effect of time dosage of BP and dosage of *Mycobacterium butyricum*. J Immunol. *113* 1958–1967 1974

Driscoll B F Kramer A J & Kies M W Myelin basic protein Location of multiple independent antigenic regions. Science *184* 73–75 1974

Dunkley P R & *Carnegie P R*. Amino acid sequence of the smaller basic protein from rat brain myelin. Biochem. J *141* 243–255 1974

Hashim G A & *Schilling, F J* Allergic encephalomyelitis Characterization of the determinants for delayed type hypersensitivity Biochem. Biophys. Res. Commun. *50* 589–596 1973

Hashim G A & *Sharpe R. D.*, Experimental allergic encephalomyelitis Structural specificity of determinants for delayed hypersensitivity Immunochem. *11* 633–640 1974

Hashim G A Hwang F & *Schilling, F J*., Experimental allergic encephalomyelitis Basic protein regions responsible for delayed type hypersensitivity Arch. Biochem. Biophys. *156* 298–309 1973

Hughes D Caspary E A & *Field E J*., Delayed hypersensitivity studies in experimental allergic encephalomyelitis a comparison of macrophage migration inhibition test and electrophoretic mobility Z. Immun. Forsch. *141* 14–19 1970

Johns E. W., Pritchard J A A Moore J L., Sutherland W H Joslin C A F Forrester J A Davies A J S., Neville A M & *Fish R G* Histones and cancer test. Nature *245* 98–99 1973

Kies M W Experimental Allergic Encephalomyelitis In *Gaull G E*. (Ed.) Biology of Brain Dysfunction. Vol. 2 Plenum Press, New York 1973 pp 185–224

Kies M W Immunology of Myelin Basic Proteins. In *Tower D B* (Ed.) The Nervous System. Vol 1 The Basic Neurosciences. Raven Press, New York 1975 pp. 637–646

Korngath S E., Kozel L. R & *Smithies O* Probable identity of tissue specific histone with encephalitogenic protein. Nature New Biology *237* 49–50 1972

Lisak R P & *Zweiman R* In vivo and in vitro immune responses to homologous myelin basic protein in experimental allergic encephalomyelitis. Cell. Immunol. *11* 212–220 1974

McDermott J R. Caspary E. A & *Dickinson, J P* Antigen cross-reactivity in the macrophage electrophoretic mobility test. A study using cellular affinity chromatography Clin. Exp Immunol. *17* 103–111 1974

Peterson P T & *Drobish, D G*., Adjuvanticity of synthetic polynucleotides for induction of experimental allergic encephalomyelitis in guinea pigs. J Immunol. *113* 194–1945, 1974

Rauch H C & *Einstein E. R*., Specific Brain Proteins: A Biochemical and Immunological Review In *Ehrenpreis S* & *Kopin I J* (Eds.) Rev Neurosciences, Raven Press, New York 1974 pp. 283–343

Schmid G., Thomas G., Lange H W & *Hempel, K* No evidence for an immunological cross reaction between encephalitogenic myelin basic protein and histones. Int. Arch. Allergy *C* 161–166 1974

Teitelbaum D., Meshorer A., Hirschfeld, T Arnon R. & *Sela M*., Suppression of experimental allergic encephalomyelitis by a synthetic polypeptide. Eur J Immunol. *1* 242–248, 1971

Vandenbark, A A Burger D R. & *Vetto R.M* Cell-mediated immunity in experimental allergic encephalomyelitis Cross reactivity between myelin basic protein and mycobacteria antigens. Proc. Soc. Exp. Biol. Med. *148* 1233–1236, 1975

Webb C., Teitelbaum D Arnon R. & *Sela M* Suppression by several synthetic polypeptides of experimental allergic encephalomyelitis induced in guinea pigs and rabbits with bovine and human basic encephalitogen. Eur J. Immunol. *3* 273–279 1973

Whitaker J The antigenicity of myelin encephalitogenic protein production of antibodies to encephalitogenic protein with deoxyribonucleic acid-encephalitogenic complexes. J Immunol. *114* 823–828, 1975

Whittingham S Bencina, B Carnegie P R & *McPherson T A*., Properties of antibodies produced in rabbits to human myelin and myelin basic protein. Int. Arch. Allergy *42* 250–263 1972.

Yamashita, U., Takami T & *Kitagawa M* Induction of anti-hapten antibody response by hapten-isologous carrier conjugate. II Specificity of hapten-reactive helper T cells. Cell. Immunol. *18* 396–410 1975

Acta path. microbiol. scand. Sect. C, 85 233–238, 1977

IN VITRO STUDIES ON NORMAL, STIMULATED AND IMMUNOLOGICALLY ACTIVATED MOUSE MACROPHAGES

I Oxidation of 1-^{14}C Glucose by Macrophages in Monolayer Cultures

S. Riisgaard, J Bennedsen and J M. Rhodes

Tuberculosis Department and WHO Collaborative Centre for Reference and Research on Escherichia, Statens Seruminstitut, Copenhagen, Denmark

Riisgaard, S., Bennedsen, J & Rhodes, J M. *In vitro* studies on normal, stimulated and immunologically activated mouse macrophages. 1 Oxidation of 1-^{14}C glucose by macrophages in monolayer cultures. Acta path. microbiol. scand. Sect. C, 85 233–238, 1977

Oxidation of 1-^{14}C glucose of BCG/listeria activated, proteose-peptone stimulated and typhoid-paratyphoid vaccine (T.A.B.) stimulated peritoneal and spleen macrophages from C3H, CBA and C57Bl mice was determined at various times after treatment. The study showed no major differences between the oxidation of stimulated and activated macrophages, but a variation was seen in C3H mice in the kinetics of glucose oxidation of BCG/listeria activated and proteose-peptone stimulated peritoneal macrophages. It was also observed, in these mice that the response appeared and disappeared earlier in the spleen than in the peritoneal macrophages.

Key words Macrophages activation *in vitro* mouse glucose oxidation.

J Bennedsen, Tuberculosis Department, Statens Seruminstitut, Amager Boulevard 80 DK 2300 Copenhagen S, Denmark

Received 3.i.77 Accepted 3.ii.77

Activation of macrophages is considered to be brought about immunologically being mediated by factors liberated from T lymphocytes. This activation usually involves an increase in microbicidal activity (14) number of lysosomes (11) content of lysosomal enzymes (11) spreading of macrophages on glass (2) phagocytosis of particles (6, 10) and oxidation of glucose through the hexose-monophosphate shunt (6, 10)

However these changes in macrophage functions might also be caused by non-specific stimulation. For example peritoneal macrophages can be stimulated *in vitro* by newborn calf serum and show an increase in content of lysosomes, lysosomal enzymes and phagocytosis of *Toxoplasma gondii* and *Listeria monocytogenes* but they are rather ineffective in restricting intracellular growth of listeria (11) Elicitation of peritoneal macrophages with proteose-peptone yields macrophages, morphologically resembling activated cells (12) which show enhanced pinocytosis (5) and increased oxidation through the hexose-monophosphate shunt (6) The question is whether activated and stimulated macrophages are essentially similar in many of their functional aspects.

It is, therefore of interest to ascertain, by *in vitro* tests, whether it is possible to distinguish between specifically activated macro-

Day E D & Pitts O M The antibody response to myelin basic protein (BP) in Lewis rats The effect of time dosage of BP and dosage of *Mycobacterium butyricum*. J Immunol. *113* 1958–1967 1974

Driscoll B F Kramer A J & Kies M W Myelin basic protein Location of multiple independent antigenic regions. Science *184* 73–75 1974

Dunkley P R & Carnegie P R Amino acid sequence of the smaller basic protein from rat brain myelin. Biochem. J *141* 243–255 1974

Hashim G A & Schilling F J Allergic encephalomyelitis Characterization of the determinants for delayed type hypersensitivity Biochem. Biophys. Res. Commun. *50* 589–596 1973

Hashim G A & Sharpe R D. Experimental allergic encephalomyelitis Structural specificity of determinants for delayed hypersensitivity Immunochem. *11* 633–640 1974

Hashim G A Hwang F & Schilling F J. Experimental allergic encephalomyelitis Basic protein regions responsible for delayed type hypersensitivity Arch. Biochem. Biophys. *156* 298–309 1973

Hughes D Caspary E A & Field E. J Delayed hypersensitivity studies in experimental allergic encephalomyelitis a comparison of macrophage migration inhibition test and electrophoretic mobility Z. Immun. Forsch. *141* 14–19 1970

Johns E W Pritchard J V A Moore J L, Sutherland W H Joslin C A F Forrester J A Davies A J S Neville A M & Fish R G Histones and cancer test. Nature *245* 98–99 1973

Kies M W Experimental Allergic Encephalomyelitis. In *Gaull G E.* (Ed.) Biology of Brain Dysfunction. Vol. 2. Plenum Press, New York 1973 pp. 185–224

Kies M W Immunology of Myelin Basic Proteins. In *Tower D B* (Ed.) The Nervous System. Vol 1 The Basic Neurosciences. Raven Press, New York 1975 pp. 637–646

Kornguth S E. Kozel L. R & Smithies O Probable identity of tissue specific histone with encephalitogenic protein. Nature New Biology *237* 49–50 1972

Lisak R P & Zweiman R In vivo and in vitro immune responses to homologous myelin basic protein in experimental allergic encephalomyelitis. Cell. Immunol *11* 212–220 1974

McDermott J R Caspary E A & Dickinson J P Antigen cross-reactivity in the macrophage electrophoretic mobility test. A study using cellular affinity chromatography Clin. Exp Immunol *17* 103–111 1974

Paterson P Y & Drobish D G. Adjuvanticity of synthetic polynucleotides for induction of experimental allergic encephalomyelitis in guinea pigs. *J Immunol. 113 1942 1946,* 1974

Rauch H C & Einstein E. R. Specific Brain Proteins A Biochemical and Immunological Review In *Ehrenpreis S & Kopin I J* (Eds.) Rev Neurosciences, Raven Press, New York 1974 pp. 283–343

Schmid G Thomas G Lange H W & Hempel, K No evidence for an immunological cross-reaction between encephalitogenic myelin basic protein and histones. Int. Arch. Allergy *47* 161–166 1974

Teitelbaum D Meshorer A, Hirschfeldt T Arnon R. & Sela M Suppression of experimental allergic encephalomyelitis by a synthetic polypeptide. *Eur J Immunol. 1 242–248,* 1971

Vandenbark A A Burger D R. & Vetto R.M Cell-mediated immunity in experimental allergic encephalomyelitis *Cross reactivity between* myelin basic protein and mycobacteria antigens. Proc. Soc. Exp. Biol. Med. *148* 1233–1236, 1975

Webb C Teitelbaum D Arnon R & Sela M Suppression by several synthetic polypeptides of experimental allergic encephalomyelitis induced in guinea pigs and rabbits with bovine and human basic encephalitogen. Eur J Immunol. *3* 273–279 1973

Whitaker J The antigenicity of myelin encephalitogenic protein production of antibodies to encephalitogenic protein with deoxyribonucleic acid-encephalitogenic complexes. J Immunol. *114* 823–828, 1975

Whittingham S Bencina, B., Carnegie P R. & McPherson T A Properties of antibodies produced in rabbits to human myelin and myelin basic protein. Int. Arch. Allergy *42* 250–263, 1972

Yamashita U Takami T & Kitagawa M Induction of anti hapten antibody response by hapten-isologous carrier conjugate II Specificity of hapten reactive helper T cells. Cell. Immunol. *18* 396-410 1975

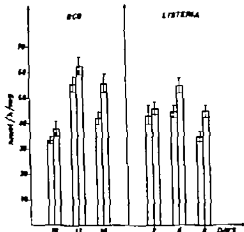


Fig. 1 Oxidation of 1-^{14}C glucose of peritoneal macrophages from C3H mice infected with BCG or Listeria. Each set of columns represents resting (left) and latex phagocytizing (right) cells. The mean with ± 2 SE of 10 cultures is shown. nmol/h/mg = nanomole/h/mg macrophage protein.

the oxidation of cells from untreated control animals (Fig. 1) (unless otherwise stated, all differences mentioned throughout this section are significant with a P value < 0.01). Cells from BCG infected mice showed the greatest increase in oxidation in both resting and phagocytizing cultures 13 days after infection. On day 16 the values were still increased, while on day 10 they were similar to control values. The ratio between the oxidation values for phagocytizing and resting cells (Ratio) only differed from the control Ratio at day 16. Cells from listeria infected mice showed the greatest increase in both values 6 days after infection, but the values were also increased on days 2 and 8. The Ratio was only increased 8 days after infection ($P < 0.02$).

The oxidation of glucose by adherent peritoneal cells from C3H mice which were injected with proteose-peptone 3, 6 or 11 days before cell harvest was compared with that of cells from untreated control mice (Fig. 2). The greatest increase in both values was found 6 days after injection. On day 3 only the phagocytizing cells showed increased oxidation and on day 11 a small decrease was noted as compared with the control. The Ratio was increased on days 3 and 6.

Fig. 2 also shows the results obtained with adherent peritoneal cells from C3H mice injected with T.A.B. 3, 5 or 8 days before cell harvest. The greatest enhancement in both values was noted on days 5 and 8, but the values were also increased on day 3. The Ratio was increased on days 5 and 8.

Oxidation values for peritoneal cells from C3H, CBA and C57B1 mice are illustrated in Fig. 3. These indicate that there are only small differences between the strains as far as untreated mice are concerned (P values from 0.03 to $\geqq 0.05$) and Ratios were all about 1.1. Compared with the values of C3H mice those of CBA mice were higher after injection with proteose-peptone (3 days) and the Ratio was further increased. Injection of T.A.B. 5 days prior to the experiments resulted in increased oxidation in all strains, CBA values being the lowest. 13 days after BCG infection the CBA values were the same as the control values. There are no data from C57B1 mice after proteose-peptone stimulation, but after T.A.B. stimulation the values equalled those from C3H mice. 13 days after BCG infection the resting value was lower in C57B1 than in C3H mice, whilst the phago-

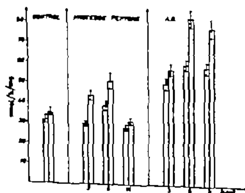


Fig. 2 Oxidation of 1-^{14}C glucose of peritoneal macrophages from C3H mice injected with proteose-peptone or T.A.B. Set of columns as in Fig. 1. The mean with ± 2 SE of 10 cultures is shown. nmol/h/mg = nanomole/h/mg macrophage protein.

phages (BCG or listeria infection) and non-specifically stimulated (proteose peptone elicited) using various criteria such as respiration digestive capacity and microbicidal activity This paper describes studies on the respiration of activated and stimulated macrophages using mouse macrophages and measuring the liberation of $^{14}CO_2$ from 1-^{14}C glucose The other aspects of these studies will be published separately (1, 13)

MATERIALS AND METHODS

Mice C3H/Ssc/1 CBA/Ssc/1 bred at Statens Seruminstitut and C57Bl/6J/BOM purchased from Bomholtgård Århus, 3–4 months old of both sexes.

Bacteria *Listeria monocytogenes* (SSC 1423) was the same strain as used previously (1) About 2×10^3 per 0 1 ml in distilled water was injected into the tail vein of each mouse 2 6 or 8 days prior to the experiments. Living lyophilized BCG vaccine constituting part of a concentrated experimental batch was obtained from the BCG Department, Statens Seruminstitut. About 10^7 viable units BCG per 0 2 ml Sauton was injected intravenously (i.v.) into each mouse 10 13 or 16 days prior to the experiments.

Stimulants 2 ml of a 10 per cent sterile solution of proteose-peptone (Difco) was injected intraperitoneally (i.p.) 3 6 or 11 days prior to the experiments. Typhoid paratyphoid vaccine (T.A.B.) was obtained from the Serum and Vaccine Department, Statens Seruminstitut. The vaccine was diluted tenfold in 0 9 per cent saline and 2 ml of this was injected i.p. 3 5 or 8 days prior to the experiments.

Preparation of macrophage monolayers Peritoneal cells were harvested from 20 mice by washing out the peritoneum with 2.5 ml Gey's solution containing 5 I U heparin/ml The cells were washed in Gey's solution containing 7.5 mmol glucose/ml (Gey-G) counted and adjusted to $2.5–5 \times 10^6$ cells/ml. 1 ml of this suspension was added to each of 20–24 screw-capped plastic tubes (NUNCLON DELTA 1409 modified with special screw-caps with pin sockets) Spleen cell monolayers were prepared from five spleens The spleens were pooled cut into small pieces, and pushed through a Borel sieve and the suspension was washed and adjusted to 1.5×10^7 cells/ml in Gey-G The monolayers were incubated for 1 hour at 37 C. Peritoneal monolayers were washed once and spleen monolayers were washed twice with Gey-G in order to remove non-adherent cells.

Oxidation of 1 ^{14}C glucose 0 1 ml of Gey-G containing 5 µCi 1 ^{14}C glucose/ml was added to each of the monolayers. In addition 0 1 ml Gey-G containing latex particles (1 mg/ml) was added to half of the cultures and Gey-G was added to all the cultures to give a final volume of 1 ml. A small wooden pin, prewrapped with filter paper was placed in the pinsocket of the screw-cap in such a manner that it did not touch the medium in the tube. The filter paper was wetted with about 0.2 ml hyamin hydroxide (New England Nuclear NEF 921) The monolayers were incubated for 1 hour at 37 C. After incubation a small hole was melted in each plastic flask, and 0.1 ml 4 N HCl was carefully placed on the inside wall without touching the medium. The hole was then covered with tape and the tubes were tilted to bring the acid into contact with the medium. 30 minutes later the pins were transferred to vials containing 15 ml scintillation solution (triton-X 100-toluol = 1 2, PPO 6 g/l, POPOP 150 mg/l) and the radioactivity was counted in a liquid scintillation counter using a quench correction estimated by the channel ration method (Nuclear of Chicago) Background controls containing only medium with radioactivity were always included.

Protein measurements. The injected acid was removed by washing twice. The monolayers were then dried and 0.5 ml 0.5 N NaOH was added to each. The monolayers were left overnight to dissolve the cells, and the protein was estimated by the method of *Lowry et al* (7) The mean value for protein found for the latex free cultures was used for the cultures containing latex.

Statistical analysis. A graphical analysis showed that the standard deviation (SD) could be approximately described by SD = 0 1 × $\bar{X}$. A normal distribution u-test was used for comparison of means. The u value was determined according to

$$u = \frac{\bar{X}_1 + \bar{X}_2}{0\,1 \frac{\bar{X}_1 n_1 + \bar{X}_2 n_2}{n_1 + n_2} \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

When comparing ratios (between phagocytizing cell oxidation and resting cell oxidation) the variance was approximated by

$$V(\ln X) = V(X)/\bar{X}^2$$

and using SD = 0 1 × $\bar{X}$ the standard error (SE) was determined according to

$$SE = 0\,1 \sqrt{\left(\frac{1}{n_{1,p}} + \frac{1}{n_1} + \frac{1}{n_{2,p}} + \frac{1}{n_2}\right)}$$

and the u value determined by ln(ratio)/SE. A two sided test was employed in all cases.

RESULTS

The oxidation of glucose by adherent peritoneal cells from C3H mice, infected with BCG 10 13 or 16 days or listeria 2 6 or 8 days before cell harvest, was compared with

the response is diminishing. This would imply that the sequence of intracellular events leading to stimulation is different from that leading to activation. Further experiments were therefore carried out with proteose-peptone elicited peritoneal macrophages from C3H mice infected with BCG 13 days before cell harvest, in order to simulate the combination of stimulation and activation in macrophages (Fig 3) This approach was unsuccessful because we observed that there was an unexpected inhibitory effect on oxidation for which we have, as yet, no explanation.

The present study demonstrates that 3 5 and 8 days after injection with T.A.B. C3H mice possessed peritoneal macrophages which showed an increase in oxidation (Fig. 2) Furthermore, this increase occurred simultaneously in both resting and phagocytizing cells, which was also seen after BCG/listeria activation.

Preliminary experiments carried out in this laboratory show that T.A.B. vaccination confers some resistance to mice against a listeria challenge as judged by survival times, and suggest that activated macrophages are present. This activation can be explained either by an immunological mechanism or by a direct effect of the T.A.B. vaccine on macrophages. The first alternative implies that the killed vaccine T.A.B. is capable of immunologically activating macrophages in a manner similar to the mechanism whereby the living BCG vaccine gives rise to activation of these cells.

Comparison of the three strains (Fig 3) shows that although responding with different intensities the general patterns are the same. One exception should be noted. When C57Bl mice are immunized with BCG the ratio between phagocytizing and resting value resembles the ratio found after treatment with T.A.B. and proteose-peptone

Enhanced oxidation by peritoneal macrophages during BCG infection in C3H mice showed a maximum about day 13 (Fig. 1) This time is coincident with the time when increased non-specific resistance measured by depression of listeria counts in the spleen and liver is maximal (4) and when BCG viable counts in the liver and spleen start to decline (4) In the same way maximum increase in oxidation during listeria infection, found here about day 6, is coincident with the time when listeria viable counts in the spleen begin to fall (8) Hence, despite the difference in development of the infections enhanced oxidation seems to be a characteristic of the activated macrophage.

Spleen macrophages show a maximum increase in ^{14}C glucose oxidation of the same magnitude as peritoneal macrophages, but this maximum is reached earlier in the infection and the response has disappeared by day 16. *North* (9) has shown that during listeria infection the unstimulated peritoneum contains mediator cells (T cells) in numbers which change in relation to the production of the cells in the spleen. But, in contrast, *Blanden* (3) cites findings showing that during ectromelia virus infection in mice, cytotoxic T cells appear in the spleen with a peak one day prior to the peak appearance of these cells in the peritoneum. The results reported here are almost easily explained by assuming a T cell distribution in the two compartments comparable to that found by *Blanden*.

The present experiments were carried out with monolayer cultures, whereas *Stubbs et al.* (15) *Karnovsky et al.* (6) and *Ratzan et al.* (10) used suspensions of peritoneal macrophages which were agitated. Those studies report that the "metabolic burst" (increase of oxidation in phagocytizing cultures) is higher than found in our study for both stimulated and activated cells. This discrepancy might be caused by the use of agitated macrophage suspensions in contrast to our use of monolayer cultures. It is a well-known fact that phagocytic cells are capable of more efficient phagocytosis in cultures which are agitated. Another noteworthy difference is the smaller variation in our experimental results from sample to sample, as expressed in the standard error which might be ascribed to the use of monolayers.

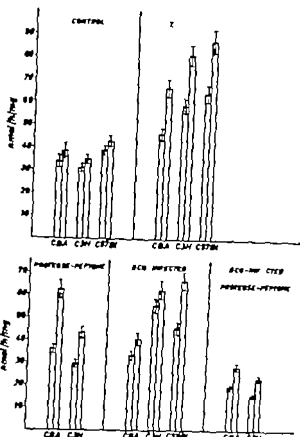


Fig 3 Oxidation of 1 ^{14}C glucose of peritoneal macrophages from C3H, CBA and C57Bl mice infected with BCG (13 days) injected with proteose-peptone (3 days) or T.A.B. (5 days) or both infected with BCG (13 days) and injected with proteose peptone (3 days) Set of colums as in Fig 1 The mean with ± 2 SE of 10 cultures is shown. nmol/h/mg = nanomole/h/mg macrophage protein.

cytizing value was about the same and consequently the Ratio was higher in this strain. There was a decrease in oxidation values in cells from CBA and C3H mice which had first been infected with BCG (13 days) and then injected with proteose peptone 3 days prior to the experiment. The Ratio however was increased in both strains.

The oxidation of adherent spleen cells from C3H mice infected with BCG 10 13 or 16 days before removal of spleens was compared with that of cells from untreated controls (Fig 4) The values for day 10 were increased, followed by a decrease on day 13 but these values were still higher than control values. A further decrease was noted on day 16 so that these values no longer differed from those of the control. The Ratio never differed from the control Ratio. Spleen cells from C3H mice injected with proteose-peptone 5 days before removal of spleens showed slightly lower values than control cells ($P < 0.05$)

DISCUSSION

The observations in this study indicate that there are no major differences between the 1-^{14}C glucose oxidation of proteose-peptone stimulated and BCG/listeria activated peritoneal macrophages from C3H mice (Figs. 1 and 2) Both stimulation and activation result in an increased oxidation of about the same magnitude and this similarity is seen in both resting and phagocytizing cells. However there is some evidence of a kinetic difference in the 1-^{14}C glucose oxidation in the stimulated and activated macrophages. In the former cells, increase in this oxidation occurs first in the phagocytizing cells appearing later in the resting cells, whereas in the activated cells the values increase simultaneously The highest ratio between phagocytizing and resting cells occurs in the activated cells when

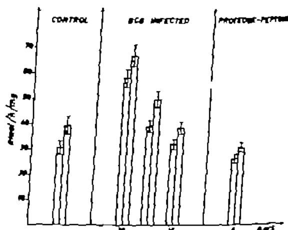


Fig 4 Oxidation of 1-^{14}C glucose of spleen macrophages from C3H mice infected with BCG or injected with proteose peptone. Set of columns as in Fig 1 The mean with ± 2 SE of 10 cultures is shown. nmol/h/mg = nanomole/h/mg macrophage protein.

the response is diminishing. This would imply that the sequence of intracellular events leading to stimulation is different from that leading to activation. Further experiments were therefore carried out with proteose-peptone elicited peritoneal macrophages from C3H mice infected with BCG 13 days before cell harvest, in order to simulate the combination of stimulation and activation in macrophages (Fig. 3). This approach was unsuccessful because we observed that there was an unexpected inhibitory effect on oxidation for which we have, as yet, no explanation.

The present study demonstrates that 3 5 and 8 days after injection with T.A.B. C3H mice possessed peritoneal macrophages which showed an increase in oxidation (Fig. 2). Furthermore, this increase occurred simultaneously in both resting and phagocytizing cells, which was also seen after BCG/listeria activation.

Preliminary experiments carried out in this laboratory show that T.A.B. vaccination confers some resistance to mice against a listeria challenge as judged by survival times, and suggest that activated macrophages are present. This activation can be explained either by an immunological mechanism or by a direct effect of the T.A.B. vaccine on macrophages. The first alternative implies that the killed vaccine T.A.B. is capable of immunologically activating macrophages in a manner similar to the mechanism, whereby the living BCG vaccine gives rise to activation of these cells.

Comparison of the three strains (Fig. 3) shows that although responding with different intensities the general patterns are the same. One exception should be noted. When C57B1 mice are immunized with BCG the ratio between phagocytizing and resting value resembles the ratio found after treatment with T.A.B. and proteose-peptone.

Enhanced oxidation by peritoneal macrophages during BCG infection in C3H mice showed a maximum about day 13 (Fig. 1). This time is coincident with the time when increased non-specific resistance, measured by depression of listeria counts in the spleen and liver is maximal (4) and when BCG viable counts in the liver and spleen start to decline (4). In the same way maximum increase in oxidation during listeria infection, found here about day 6, is coincident with the time when listeria viable counts in the spleen begin to fall (8). Hence, despite the difference in development of the infections enhanced oxidation seems to be a characteristic of the activated macrophage.

Spleen macrophages show a maximum increase in 1 C glucose oxidation of the same magnitude as peritoneal macrophages, but this maximum is reached earlier in the infection and the response has disappeared by day 16. *North* (9) has shown that during listeria infection the unstimulated peritoneum contains mediator cells (T cells) in numbers which change in relation to the production of the cells in the spleen. But, in contrast, *Blanden* (3) cites findings showing that during ectromelia virus infection in mice cytotoxic T cells appear in the spleen with a peak one day prior to the peak appearance of these cells in the peritoneum. The results reported here are almost easily explained by assuming a T cell distribution in the two compartments comparable to that found by *Blanden*.

The present experiments were carried out with monolayer cultures, whereas *Stubbs et al.* (15), *Karnovsky et al.* (6) and *Ratzan et al.* (10) used suspensions of peritoneal macrophages which were agitated. Those studies report that the "metabolic burst" (increase of oxidation in phagocytizing cultures) is higher than found in our study for both stimulated and activated cells. This discrepancy might be caused by the use of agitated macrophage suspensions in contrast to our use of monolayer cultures. It is a well-known fact that phagocytic cells are capable of more efficient phagocytosis in cultures which are agitated. Another noteworthy difference is the smaller variation in our experimental results from sample to sample, as expressed in the standard error which might be ascribed to the use of monolayers.

We would like to acknowledge the expert secretarial assistance of *A Thorborg* and the excellent technical assistance of *A G Overgaard* for the photographic work.

REFERENCES

1. *Bennedsen J Rusgaard S Rhodes J M & Olesen Larsen S* *In vitro* studies on normal stimulated and immunologically activated mouse macrophages. III Intracellular multiplication of *Listeria monocytogenes*. Acta path. scand Sect. C *85* 246–252 1977
2. *Blanden R V* Modification of macrophage function. J reticuloendothel. Soc. *5* 179–202 1968
3. *Blanden R V* T-cell response to viral and bacterial infection. Transplant. Rev *19* 56–88 1974
4. *Blanden R V Lefford M J & Mackaness G B* The host response to Calmette-Guérin Bacillus infection in mice. J exp Med *129* 1079–1107 1969
5. *Edelson P J Stubbel R & Cohn Z A* The pinocytic rate of activated macrophages. J exp Med. *142* 1150–1164 1975
6. *Karnovsky M L., Lazdins J & Simmons S R* Metabolism of activated mononuclear phagocytes at rest and during phagocytosis. In *van Furth R* (Ed) Mononuclear Phagocytes in Immunity Infection and Pathology Blackwell Scient. Publ. Oxford 1975 p. 423–439
7. *Lowry O H Rosebrough N J Farr A L. & Randall R J* Protein measurement with the folin phenol reagent. J biol. Chem. *193* 265–275 1951
8. *North R J* Cellular kinetics associated with the development of acquired cellular resistance J exp. Med. *130* 299–311 1969
9. *North R J* T-cell-dependent macrophage activation in cell mediated anti-Listeria immunity In *Wagner W-H Hahn H & Evans R.* (Eds) Activation of Macrophages. Excerpta Medica, Amsterdam 1974
10. *Ratzan K R Musher D M., Keusch, G. T & Weinstein L.* Correlation of increased metabolic activity resistance to infection, enhanced phagocytosis, and inhibition of bacterial growth by macrophages from Listeria- and BCG-infected mice. Infect. Immun. *5* 499–504 1972.
11. *Reikvam A., Gammeltvedt R & Heiby E.A.* Activated mouse macrophages morphology lysosomal biochemistry and microbicidal properties of *in vivo* and *in vitro* activated cells Acta path. microbiol. scand. Sect. C, *83* 129–138, 1975
12. *Rhodes J M* *In vitro* studies on the fate of antigen. 5 The uptake of I^{131} HSA by mouse exudate cells in the presence of normal serum and anti-HSA serum. Acta path. microbiol. scand. Sect. *65* 457–466 1965
13. *Rhodes J M Nielsen G Olesen Larsen S Bennedsen J & Rusgaard S* *In vitro* studies on normal, stimulated and immunologically activated mouse macrophages. II Degradation of radioactive antigen/antibody complexes. Acta path. microbiol. scand. Sect. C, *85* 239–245 1977
14. *Simon H B & Sheagren J N* Enhancement of macrophage bactericidal capacity by antigenically stimulated immune lymphocytes. Cell Immunol. *4* 163–174 1972.
15. *Stubbs M., Kühner A. V., Glass E. A., David J R. & Karnovsky M L.* Metabolic and functional studies on activated mouse macrophages. J exp Med. *137* 537–542, 1973.

Acta path. microbiol. scand. Sect. C, 85 239-245 1977

IN VITRO STUDIES ON NORMAL, STIMULATED AND IMMUNOLOGICALLY ACTIVATED MOUSE MACROPHAGES

II Degradation of Radioactive Antigen/Antibody Complexes

J M Rhodes, G Nielsen, S Olesen Larsen
J Bennedsen and S. Riisgaard

WHO Collaborative Centre for Reference and Research on Escherichia,
Biostatistical and Tuberculosis Departments, Statens Seruminstitut, Copenhagen, Denmark

Rhodes, J M., Nielsen, G., Olesen Larsen, S Bennedsen, J & Riisgaard, S *In vitro studies* on normal, stimulated and immunologically activated mouse macrophages II. Degradation of radioactive antigen/antibody complexes. Acta path. mikrobiol. scand. Sect. C, 85 239-245 1977

Radioactive antigen/antibody complexes are degraded to a greater extent by PE macrophages from C3H, CBA and C57Bl mice injected intraperitoneally with proteose-peptone (stimulated macrophages) and T.A.B vaccine than by PE macrophages from normal mice and mice immunized with BCG (activated macrophages) On the other hand, spleen macrophages from mice immunized with BCG had a greater digestive capacity than macrophages from mice injected with proteose-peptone or T.A.B. vaccine or from normal mice. Thus, activated macrophages are capable of degrading antigen/antibody complexes, provided that the cells are harvested from one of the foci of BCG infection.

Key words Macrophage activation *in vitro* mouse degradation.

J M. Rhodes, Collaborative Centre for Reference and Research on Escherichia (WHO) Statens Seruminstitut, Amager Boulevard 80, DK 2300 Copenhagen S Denmark.

Received 3 77 Accepted 3.ii 77

In a previous paper (14) we have described studies on the respiration of activated and stimulated mouse macrophages. The only difference which was observed in the metabolism of the two types of cells was a kinetic difference, and thus the measurement of respiration could not be employed to distinguish these cells. We therefore turned our attention to another functional aspect of macrophages, namely digestive capacity in order to ascertain whether the assay of this function could detect any difference between stimulated and activated macrophages.

It has been shown that non-specifically stimulated macrophages have a greater capacity for phagocytosis than non-stimulated macrophages. Furthermore, the former type of cell has a greater content of hydrolases than the latter and as a consequence a greater capacity to degrade proteins (3) There is also evidence (reviewed in ref. 1) that immunologically activated macrophages contain larger amounts of hydrolases than normal

macrophages. In addition, it has been shown conclusively that activated macrophages have a greater bactericidal effect against intracellular facultative parasites when compared with normal macrophages (12), but there is no clear evidence that proteolytic enzymes are responsible for this effect.

In view of these findings, it was of interest to determine whether stimulated and activated macrophages differ in their capacity to degrade particulate matter and whether this digestive capacity could be correlated with their ability to inhibit the growth of intracellular *Listeria monocytogenes* (see subsequent article)

This paper presents studies on the ability of normal stimulated and activated macrophages derived from the peritoneum and spleen of three different strains of mice to degrade radioactive antigen/antibody complexes.

MATERIALS AND METHODS

Mice C3H/Ssc/1 female, CBA/Ssc/1 male bred at the Statens Seruminstitut, and C57Bl/6J/BOM female purchased from Bomholtgård, Århus. All were 3–4 months old.

Bacteria. About 10^7 viable units BCG in 0.2 ml Sauton were injected intravenously (i v) into each mouse 7 12 and 16 days prior to the experiments as described previously (4)

Stimulants. 2 ml of a 10 per cent sterile solution of proteose/peptone (Difco) (PP) was injected intraperitoneally (i p) 3 days prior to the experiments. *Typhoid paratyphoid vaccine* (T.A.B.) was obtained from the Serum and Vaccine Department Statens Seruminstitut The vaccine was diluted ten fold in 0 9 per cent saline and 1 ml of this was injected i.p 5 and 10 days prior to the experiments.

Carrier free ^{125}I was purchased from the Radiochemical Centre Bucks, England.

Human serum albumin (HSA) was obtained from the Blood Fractionation Department Statens Seruminstitut. HSA was labelled at a specific activity of about 0.5 μc/μg according to *Hunter & Greenwood* (8)

Anti HSA serum pool (batch 82) Twenty rabbits were injected twice subcutaneously with an emulsion of 0 1 mg HSA/0.5 ml saline with 0.5 ml Freund's incomplete adjuvant with a monthly interval between each injection. Only sera with high precipitating titres were used for the pool.

Latex suspension (Bacto-Latex, Difco Laboratory Detroit, USA) 0 1 ml latex was mixed with 9.9 ml medium (Eagle's minimal essential medium (MEM) containing 10 per cent foetal calf serum and centrifuged at 2000 *g* for 15 minutes to remove any aggregates.

Acridine orange was purchased from Fluka and was used as a 0 01 per cent solution in MEM containing 10 per cent foetal calf serum.

Degradation of ^{125}I *HSA/Antibody Complexes by Peritoneal Exudate (PE) Cells*

Ten to 20 mice were used for the experiments PE cells were harvested by washing out the peritoneum with 3 ml saline containing 15 IU heparin and 5 per cent normal rabbit serum. All cells were pooled, centrifuged at 560 *g* for 5 minutes and then washed twice with and re-suspended in Gey's solution to a concentration of 10, 20, 30, 40, or 50 × 10^6 cells/ml, depending on the total number of available cells. 0 8 ml Gey's solution was placed in each of 10 tubes (NUNC 63 × 15 mm) for each series of cells, together with 0 1 ml anti-HSA serum and 0.1 ml of the appropriate cell suspension and 10 μg ^{125}I HSA/20 μl. Controls consisted of 10 tubes containing all ingredients except the cells. The radioactivity in each tube was measured before incubation All tubes were incubated at 37° C for 3 hours with frequent shaking. 0.2 ml of 25 per cent trichloracetic acid (TCA) was added to each tube after incubation and the mixtures were centrifuged at 940 *g* for 10 minutes and the radioactivity in the supernatant was measured. Evaluation of the breakdown of ^{125}I-labelled HSA/antibody complexes is based on the non-protein bound radioactivity in the supernatant calculated as a percentage of the original radioactivity added.

Degradation of ^{125}I *HSA/Antibody Complexes by Spleen Cells*

Spleens were removed from each group of mice after harvesting the PE cells. Six to seven spleens were used for each experiment. The spleens were cut into small pieces and pushed through a Borel sieve into about 10 ml Gey's solution. The cells were centrifuged at 380 *g* re-suspended in Gey's solution and counted. The degradation was carried out as above, except that 10 times as many cells were placed in each of 10 tubes.

Determination of the Percentage Macrophages in PE and Spleen Cells

Functional tests as described by *Hertel Wulff & Rubin* (7)

a) Incubation of cells with latex for 30 minutes at 37 C. The cells were then washed with Gey's solution and cytocentrifuged Cells which had ingested 10 or more latex particles were denoted macrophages.

TABLE 1. *Degradation of ^{131}I-HSA/Antibody Complexes by PE Cells from C3H Mice*

Treatment	Per cent M	$\times 10^6$ PE cells				
		1	2	3	4	5
Normal	29.6	2.8	4.5	7.4		12.4
PP 5 days	35.4	11.1	19.0		32.0	35.1
T.A.B 5 days	35.9	5.1	12.0		24.5	30.5
T.A.B 10 days	27.6	1.2		3.7		9.9
BCG 7 days	30.4	1.1		3.7		6.1
BCG 12 days A	33.6	2.0		3.0		7.0
BCG 12 days B	40.0	4.5		11.7		17.5
BCG 16 days	43.6	2.3		6.9		10.6

M = macrophages (average of three tests, see text)
PE = peritoneal cells.
PP = proteose/peptone.
PP and T.A.B injected intraperitoneally
BCG injected intravenously

b) Incubation of cells with acridine orange 1:20 for 30 minutes at 37° C. The cells were washed once with Gey' solution and cytocentrifuged. The cells were examined in the fluorescence microscope and those containing 8 or more lysosomes were designated macrophages.

c) Morphologically after cytocentrifugation and staining with Wright's stain.

A total of 1000 cells was counted for each preparation.

RESULTS

Degradation of ^{131}I-HSA/Antibody Complexes by PE Cells

The basic data for C3H mice are shown in Table 1 illustrating the type of results obtained with all three strains of mice. The three tests used for assessing the number of macrophages in PE cells were in good agreement and the average value was used for correction of the percentage degradation (see below). PE macrophages harvested from mice immunized with BCG were much larger than control macrophages and resembled the stimulated cells, i.e. both had increased cytoplasm and a larger number of vacuoles. All these groups of mice possessed varying percentages of macrophages in their PE cells and thus it is not strictly correct to compare the degradation of complexes by whole PE cell populations. Degradation was therefore expressed as the percentage degradation per 10^6 *macrophages* in each PE cell population. These results are illustrated in Fig. 1. Preliminary experiments carried out after immunization with BCG had shown that there was a variability in the percentage degradation of complexes by PE macrophages from C3H and CBA mice. This variation is illustrated in Table 1 (BCG 12 days A+B). In this instance it was decided to use the average values for the two experiments as a basis for the calculation presented in Fig. 1 for BCG (12 days) for C3H and CBA mice, to equalize this variation. Fig. 1 shows that PE macrophages obtained after i.p. injection of PP and T.A.B (5 days) degraded complexes to a greater extent than any of the other groups of cells, including control cells, in all strains of mice. All mice injected with PP showed a decreased degradation per 10^6 macrophages as the number of PE cells increased, which may be due to overactive cells being overcrowded in the medium. In contrast, 10^6 macrophages obtained after injection with T.A.B (5 days) degraded complexes to the same extent, irrespective of the number of PE cells present, whereas 10 days after injection with T.A.B. there seemed to be an increase in degradation by 10^6 macrophages with increase in the number of PE cells. Cells obtained from all strains of mice after immunization with BCG (7, 12 and 16 days)

macrophages. In addition, it has been shown conclusively that activated macrophages have a greater bactericidal effect against intracellular facultative parasites when compared with normal macrophages (12), but there is no clear evidence that proteolytic enzymes are responsible for this effect.

In view of these findings it was of interest to determine whether stimulated and activated macrophages differ in their capacity to degrade particulate matter and whether this digestive capacity could be correlated with their ability to inhibit the growth of intra cellular *Listeria monocytogenes* (see subsequent article)

This paper presents studies on the ability of normal stimulated and activated macrophages derived from the peritoneum and spleen of three different strains of mice to degrade radioactive antigen/antibody complexes.

MATERIALS AND METHODS

Mice C3H/Ssc/1 female CBA/Ssc/1 male bred at the Statens Seruminstitut, and C57Bl/6J/BOM, female purchased from Bomholtgård Århus. All were 3–4 months old.

Bacteria. About 10^7 viable units BCG in 0.2 ml Sauton were injected intravenously (i.v.) into each mouse 7 12 and 16 days prior to the experiments as described previously (4)

Stimulants 2 ml of a 10 per cent sterile solution of proteose peptone (Difco) (PP) was injected intraperitoneally (i.p.) 3 days prior to the experiments. Typhoid paratyphoid vaccine (T.A.B.) was obtained from the Serum and Vaccine Department, Statens Seruminstitut. The vaccine was diluted ten fold in 0.9 per cent saline and 1 ml of this was injected i.p. 5 and 10 days prior to the experiments.

Carrier free ^{125}I was purchased from the Radiochemical Centre Bucks, England

Human serum albumin (HSA) was obtained from the Blood Fractionation Department, Statens Seruminstitut. HSA was labelled at a specific activity of about 0.5 $\mu c/\mu g$, according to *Hunter & Greenwood* (8)

Anti-HSA serum pool (batch 82) Twenty rabbits were injected twice subcutaneously with an emulsion of 0.1 mg HSA/0.5 ml saline with 0.5 ml Freund's incomplete adjuvant with a monthly interval between each injection. Only sera with high precipitating titres were used for the pool.

Latex suspension (Bacto-Latex, Difco Laboratory Detroit, USA) 0.1 ml latex was mixed with 9.9 ml medium (Eagle's minimal essential medium (MEM) containing 10 per cent foetal calf serum and centrifuged at 2000 g for 15 minutes to remove any aggregates.

Acridine orange was purchased from Fluka and was used as a 0.01 per cent solution in MEM containing 10 per cent foetal calf serum.

Degradation of ^{125}I *HSA/Antibody Complexes by Peritoneal Exudate (PE) Cells*

Ten to 20 mice were used for the experiments PE cells were harvested by washing out the peritoneum with 3 ml saline containing 15 IU heparin and 5 per cent normal rabbit serum. All cells were pooled, centrifuged at 560 g for 5 minutes and then washed twice with and re-suspended in Gey's solution to a concentration of 10 20 30, 40, or 50 × 10^6 cells/ml, depending on the total number of available cells. 0.8 ml Gey's solution was placed in each of 10 tubes (NUNC 63 × 15 mm) for each series of cells, together with 0.1 ml anti-HSA serum and 0.1 ml of the appropriate cell suspension and 10 μg ^{125}I HSA/20 μl. Controls consisted of 10 tubes containing all ingredients except the cells. The radioactivity in each tube was measured before incubation. All tubes were incubated at 37 C for 3 hours with frequent shaking 0.2 ml of 25 per cent trichloracetic acid (TCA) was added to each tube after incubation and the mixtures were centrifuged at 940 g for 10 minutes and the radioactivity in the supernatant was measured. Evaluation of the breakdown of ^{125}I-labelled HSA/antibody complexes is based on the non-protein bound radioactivity in the supernatant calculated as a percentage of the original radioactivity added.

Degradation of ^{125}I *HSA/Antibody Complexes by Spleen Cells*

Spleens were removed from each group of mice after harvesting the PE cells Six to seven spleens were used for each experiment. The spleens were cut into small pieces and pushed through a Borel sieve into about 10 ml Gey's solution. The cells were centrifuged at 560 g re-suspended in Gey's solution and counted. The degradation was carried out as above, except that 10 times as many cells were placed in each of 10 tubes.

Determination of the Percentage Macrophages in PE and Spleen Cells

Functional tests as described by *Hertel Wulff & Rubin* (7)

a) Incubation of cells with latex for 30 minutes at 37 C. The cells were then washed with Gey's solution and cytocentrifuged. Cells which had ingested 10 or more latex particles were denoted macrophages.

TABLE 2. *Degradation of ^{131}I-HSA/Antibody Complexes by Spleen Cells from C3H Mice*

Treatment	Per cent M	× 10⁷ spleen cells		
		1	3	5
Normal	3.7	0.55	2.2	3.2
PP 3 days	3.6	1.4	3.9	5.4
T.A.B 5 days	3.2	1.3	3.7	5.3
T.A.B 10 days	5.0	0.8	2.6	4.2
BCG 7 days	4.0	1.3	2.8	3.9
BCG 12 days	5.7	2.1	4.8	6.6
BCG 16 days	3.6	3.7	6.2	6.3

For explanation of symbols see Table 1

mouse (7 days) as compared with C3H mice (16 days)

Evaluation of results

The standard deviation corresponding to the tube to tube variation was rather small, averaging 10–15 per cent of the mean degradation, so that the standard error due to this variation (standard deviation/$\sqrt{10}$) is of minor importance. The effect of the mouse to mouse variation is thought to be negligible because the cells used *in each experiment* were pooled for at least 10 inbred mice. Other factors which might be considered in a statistical evaluation are variation due to the immunization/stimulation procedure and errors in the determination of the percentage of macrophages. These factors cannot be elucidated from the present results.

The results obtained after immunization with BCG showed a considerable variation from experiment to experiment (see BCG 12 days A+B, C3H mice, Table 1). The BCG vaccine was derived from one batch and therefore viability and potency were assumed to be constant. The concentration of living organisms was controlled by a plating technique. Only a small variation was found in the other groups when the results were compared with those from preliminary experiments *not illustrated in this paper*.

DISCUSSION

The present studies have shown that activated and stimulated macrophages have a greater digestive capacity than cells from normal mice. However there are some interesting inconsistencies in the results which will be discussed in turn.

Mice stimulated intraperitoneally with PP yield macrophages which have an enhanced digestive capacity. This is also the case 5 days after i.p. injection with T.B.A. vaccine, but 10 days after injection with this vaccine PE macrophages degraded antigen/antibody complexes to about the same extent as normal macrophages. Immunization with BCG did not give rise to an increased degradation in PE macrophages. This is an interesting point because these macrophages showed various signs of activation such as increased spreading, larger size, and increased content of vesicles as compared with cells from normal mice (5). They also appeared to resemble PP stimulated macrophages morphologically. Despite indications that these cells were activated there is no real evidence that PE macrophages from mice immunized with BCG perform better than normal cells (Fig. 1). We feel that this conclusion is justified because we have found that PE macrophages from mice immunized with BCG show a greater variability from experiment to experiment, in their digestive capacity than the PE macrophages *in any* of the other groups.

The assay used for the present studies detects enzyme activity amongst these being cathepsin, which is known to degrade HSA (10). I.p. injection of antigen and other stimulants causes an increase in proteolytic en-

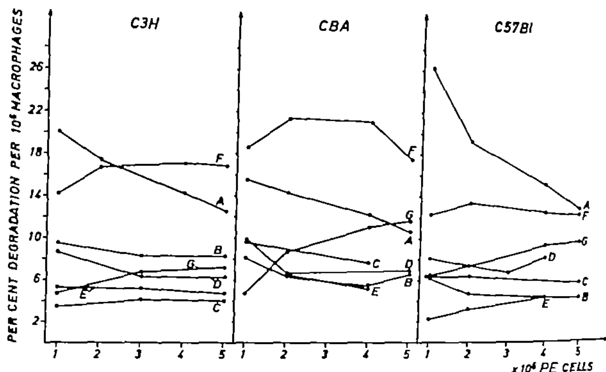


Fig 1 Degradation of ^{125}I HSA/anti HSA complexes by PE macrophages from mice treated in different ways prior to cell harvest. Degradation is expressed as the percentage degradation per *10^6 macrophages* in 1–5 × 10^6 PE cells. Each point represents the average of 10 simultaneous determinations. A = proteose-peptone 3 days after injection, B = control, C = BCG 7 days after immunization, D = BCG 12 days after immunization E = BCG 16 days after immunization, F = T.A.B. vaccine 5 days after immunization G = T.A.B vaccine 10 days after immunization.

degraded complexes to more or less the same extent as normal cells, irrespective of the number of PE cells present.

Degradation of ^{125}I HSA/Antibody Complexes by Mouse Spleen Cells

The three tests used for assessing the number of macrophages in the spleen cell suspensions were not in good agreement. Cell counts were much higher after staining with Wright's stain and uptake of acridine orange which may have been due to the inclusion of cells which did not ingest latex but morphologically resembled mononuclear phagocytic cells. We decided to assess the percentage of macrophages by means of the latex test since the phagocytic cells only were important for these experiments.

The percentage of macrophages was similar in all groups of mice with the exception of those immunized with BCG (Table 2 which shows the results with C3H mice). In these mice the macrophages were larger and had many more vesicles than macrophages from normal mice, although the activated cells appeared to be morphologically somewhat similar to stimulated macrophages.

Table 2 shows the basic data for the degradation of complexes by spleen cells from the three strains of mice. These results are also expressed as the percentage degradation per *10^6 macrophages* and are shown in Fig 2. Cells harvested from C3H mice immunized with BCG (16 days) were far superior to any of the cells in the other groups as far as digestive capacity was concerned. On the other hand 10^6 macrophages from all groups of CBA mice tended to degrade complexes to the same extent irrespective of the number of spleen cells present. Cells from C57Bl mice immunized with BCG (7 and 12 days) degraded complexes to a greater extent than cells from the other groups. The peak of degradation is thus earlier in this strain of

for the above supposition from the experiments of *Lnke & Hahn* (11) who found that activation of macrophages *in vivo* is only brought about when a specific immunological reaction occurs in their immediate vicinity

Maximum increase in digestive capacity in the spleen cells from BCG immunized mice was from 7–16 days depending on the strain of mouse (see Fig 2) This agrees with the time of onset of non-specific resistance to listeria (13) and the enhanced oxidation by peritoneal macrophages (14)

Although our results have demonstrated that activated and stimulated macrophages are capable of degrading complexes they tell us nothing about the enzymes which may be involved in bactericidal killing or the actual difference if any between these two types of cells as far as this functional aspect is concerned. However *Dannenberg et al.* (6) have published results which suggest that not every lysosomal enzyme is increased to the same degree in activated macrophages, but that the increase in β-galactosidase might be an expression of immunological activation. *Ando et al* (2) have recently shown that PHA stimulated lymphocytes and whole culture supernatants from these cells increased the adherence of rabbit PE cells, and that morphologically the two sets of PE cells resembled each other but that β-galactosidase activity was only induced in PE macrophages by the activated lymphocytes and not by their supernatants. It is of importance to know whether this enzyme is only increased in activated macrophages and not in stimulated macrophages because this would imply that a direct membrane interaction between lymphocyte and macrophage is required as a triggering mechanism for the activation of the latter cell. Experiments are in progress in our laboratories to elucidate this point.

We would like to acknowledge the expert secretarial assistance of *A Thorberg* and the excellent technical assistance of *B Melander Nielsen* and *A. O. Overgaard* for the photographic work

REFERENCES

1 *Allison A C & Davie P.*, Increased biochemical and biological activities of mononuclear phagocytes exposed to various stimuli, with special reference to secretion of lysosomal enzymes. In: *van Furth R.*, Mononuclear Phagocytes in Immunity Infection and Pathology Blackwell Scient. Publ., Oxford 1975 p. 487–506.

2. *Ando M., Suga M., Shima K., Sugimoto M., Higuchi S., Tsuda, T & Tokomoto H.*, Different effects of phytohemagglutinin-activated lymphocytes and their culture supernatants on macrophage function. Infect. Immun. *13* 1442–1448, 1976

3 *Axline S G & Cohn Z.*, *In vitro* induction of lysosomal enzymes by phagocytosis. J exp. Med. *131* 1239–1260 1970

4 *Bennedsen, J & Olesen Larsen S.*: The BCG-induced resistance to listeriosis. Acta path. microbiol. scand Sect. C, *83* 377–382, 1975.

5. *Blanden R. V.*, Modification of macrophage function. J reticuloendoth. Soc. *5* 179–202, 1968.

6. *Dannenberg, A. M Meyer O T., Esterly J R. & Kambara T* The local nature of immunity in tuberculosis, illustrated histochemically in dermal BCG lesions. J Immunol. *5* 931–941 1968

7 *Hertel-Wulff B. & Rubin B* Lymphocyte proliferation *in vitro* induced by soluble protein antigens. II Cellular requirements. Europ. J Immunol. *6* 418–424 1976.

8. *Hunter W M & Greenwood F C.*, Preparation of iodine-131 labelled human growth hormone of high specific activity N ture (Lond.) *194* 495–496 1962.

9 *Kolukov D & Rhodes J M.*, Effect of antigenic stimulation on the enzymatic activity of mouse peritoneal and spleen cells. Scand. J Immunol. 2 97–106 1973

10. *Lapresle C & Webb T.*: Study of a proteolytic enzyme from rabbit spleen. Biochem. J. *76* 538–543 1960.

11 *Lisk T & Hahn H* Die immunologisch induzierte Steigerung der Abtötungsleistung von Peritonealmakrophagen der Maus gegen *Listeria monocytogenes* Zbl. Bakt. Hyg. I. Abt. Orig. *A 235* 147–152, 1976.

12 *Mackaness, G* Cellular immunity In *van Furth R.*, Mononuclear Phagocytes. Blackwell Scient. Publ., Oxford 1970 p. 461–475

13. *Rhodes J M., Bennedsen J & Olesen Larsen S.*: Unpublished observations.

14 *Rdisgaard S Bennedsen J & Rhodes, J M.*, *In vitro* studies on normal, stimulated and immunologically activated mouse macrophages. I. Oxidation of 1-¹⁴C glucose of macrophages in monolayer cultures. Acta path. microbiol. scand. Sect. C, *85* 233–238, 1977

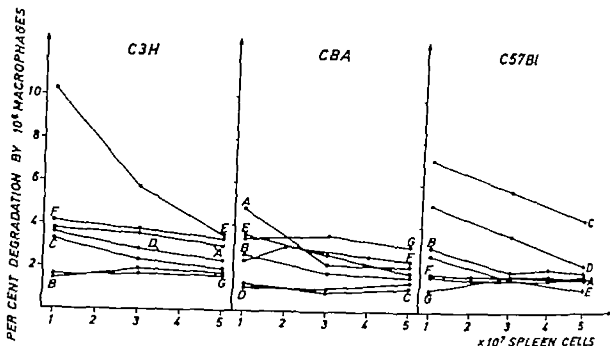


Fig 2 Degradation of ^{125}I HSA/anti HSA complexes by spleen macrophages from mice treated in different ways prior to cell harvest. Degradation is expressed as *the percentage degradation per 10^6 macrophages* in 1–5 × 10^7 spleen cells. Each point represents the average of 10 simultaneous determinations. For explanation of symbols, see Fig. 1

zymes in PE macrophages but this effect is only transient since the content of enzymes declines after 7 days (3 9) The results obtained with PE macrophages from mice injected with PP or T.A.B vaccine can thus be explained by this increase and decrease in the amount of enzyme available

Rhodes et al (13) have observed that mice are resistant to listeria 10 days after injection of T.A.B vaccine and that non-specific resistance is also observed at about the same time *after primary immunization with BCG* According to *Mackaness* (12) activated macrophages are present at the onset of resistance and would certainly be found 10–12 days after immunization with BCG However PE macrophages harvested from mice treated with T.A.B. or BCG showed no enhanced capacity to degrade complexes at this period The state of immunity induced by BCG and T.A.B. about 10 days after injection although not detected by a test for digestive activity does affect PE macrophages to the extent that they show an increased 1-^{14}C glucose oxidation (*14*) *This suggests two possibilities* a) proteolytic enzymes which degrade complexes in PE stimulated macrophages are not involved in bactericidal killing and b) the *threshold for activating digestive capacity is* greater than that required for activation of increased respiration (14)

In contrast to the results obtained with PE macrophages, cells taken from a focus of infection *i.e* BCG activated spleen cells—from C3H and C57Bl mice—degrade complexes to a greater extent than those from any of the other groups. Since *BCG is injected i.v it* multiplies in the spleen thus ensuring that antigen is continuously present for interaction with lymphocytes and macrophages resulting in the activation of the latter cells (12) This suggests that sufficient antigen and/or immunocompetent cells are not present in the peritoneum for the activation or direct stimulation of macrophages which would explain why PE macrophages from BCG immunized mice behave like normal cells. On the other hand, sufficient antigen must be present to cause the altered morphological appearance of the macrophages. There is some evidence

for the above supposition from the experiments of *Linke & Hahn* (11) who found that activation of macrophages *in vivo* is only brought about when a specific immunological reaction occurs in their immediate vicinity

Maximum increase in digestive capacity in the spleen cells from BCG immunized mice was from 7–16 days depending on the strain of mouse (see Fig. 2) This agrees with the time of onset of non-specific resistance to listeria (13) and the enhanced oxidation by peritoneal macrophages (14)

Although our results have demonstrated that activated and stimulated macrophages are capable of degrading complexes they tell us nothing about the enzymes which may be involved in bactericidal killing or the actual difference, if any between these two types of cells as far as this functional aspect is concerned. However *Dannenberg et al.* (6) have published results which suggest that not every lysosomal enzyme is increased to the same degree in activated macrophages, but that the increase in β-galactosidase might be an expression of immunological activation. *Ando et al.* (2) have recently shown that PHA stimulated lymphocytes and whole culture supernatants from these cells increased the adherence of rabbit PE cells, and that morphologically the two sets of PE cells resembled each other but that β-galactosidase activity was only induced in PE macrophages by the activated lymphocytes and not by their supernatants. It is of importance to know whether this enzyme is only increased in activated macrophages and not in stimulated macrophages because this would imply that a direct membrane interaction between lymphocyte and macrophage is required as a triggering mechanism for the activation of the latter cell. Experiments are in progress in our laboratories to elucidate this point.

W ould like to acknowledge the expert secretarial assistance of *A Thorberg* and the excellent technical assistance of *B Melander Nielsen* and *A G Overgaard* for the photographic work.

REFERENCES

1 *Allison A C & Davies, P.* Increased biochemical and biological activities of mononuclear phagocytes exposed to various stimuli, with special reference to secretion of lysosomal enzymes. In *van Furth R.* Mononuclear Phagocytes in Immunity Infection and Pathology Blackwell Scient. Publ., Oxford 1975 p. 487–506.

2 *Ando M., S ga, M Shima K., S gimot M Higuchi S Trada T & Takanoi H* Different effects of phytohemaggl tinin-activated lymphocytes and their culture supernatants on macrophage function. Infect. Immun. *13* 1442–1448, 1976.

3 *Axline S G. & Cohn Z., I vitro* Induction of lysosomal enzymes by phagocytosis. J exp. Med. *131* 1239–1260 1970.

4 *Bennedsen, J & Olesen Larsen S.:* The BCG-induced resistance to listeriosis. Acta path. microbiol. scand Sect. C, *83* 377–382, 1975

5 *Blanden R. V* Modification of macrophage function. J reticuloendoth, Soc. *5* 179–202 1968.

6 *Dannenberg, A. M., Meyer O T., Esterly J R & Kambara, T* The local nature of immunity in tuberculosis, illustrated histochemically in dermal BCG lesions. J Immunol. *5* 931–941 1968.

7 *Hertel-Wulff B. & Rubin, B.* Lymphocyte proliferation *in vitro* induced by soluble protein antigens. II. Cellular requirements. Europ. J Immunol. *6* 418–424 1976

8. *Hunter W M & Greenwood F C.* Preparation of iodine-131 labelled human growth hormone of high specific activity Nature (Lond.) *194* 495–496 1962.

9 *Kotulova, D & Rhodes J M* Effect of antigenic stimulation on the enzymatic activity of mouse peritoneal and spleen cells. Scand. J Immunol. *2* 97–106, 1973.

10 *Lapresle C & Webb T.:* Study of proteolytic enzyme from rabbit spleen. Biochem. J *76* 538–543 1960.

11. *Linke T & Hahn, H.* Die immunologisch induzierte Steigerung der Abtötungsleistung on Peritonealmakrophagen der Maus gegen *Listeria monocytogenes.* Zbl. Bakt. Hyg. I. Abt. Orig. *A 235* 147–152, 1976.

12. *Mackaness G* Cellular Immunity In: *van Furth R.* Mononuclear Phagocytes. Blackwell Scient. Publ Oxford 1970 p. 461–475.

13. *Rhodes, J M Bennedsen J & Olesen Larsen S.* Unpublished observations

14 *Riisgaard S Bennedsen J & Rhodes, J M.* In vitro studies on normal, stimulated and immunologically activated mouse macrophages I Oxidation of 1-¹⁴C glucose of macrophages in monolayer cultures. Acta path. microbiol. scand. Sect. C, *85* 233–238, 1977

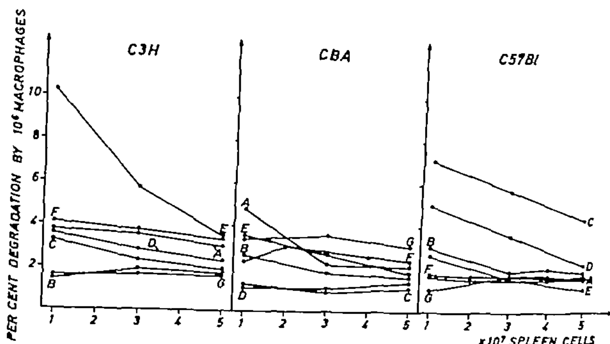


Fig. 2 Degradation of ¹²⁵I HSA/anti-HSA complexes by spleen macrophages from mice treated in different ways prior to cell harvest. Degradation is expressed as the percentage degradation per *10⁶ macrophages* in 1–5 × 10⁷ spleen cells. Each point represents the average of 10 simultaneous determinations. For explanation of symbols, see Fig. 1

zymes in PE macrophages, but this effect is only transient since the content of enzymes declines after 7 days (3 9) The results obtained with PE macrophages from mice injected with *PP* or *T.A.B* vaccine can thus be explained by this increase and decrease in the amount of enzyme available

Rhodes et al (13) have observed that mice are resistant to listeria 10 days after injection of T.A.B vaccine and that non specific resistance is also observed at about the same time after primary immunization with BCG According to *Mackaness* (12) activated macrophages are present at the onset of resistance and would certainly be found 10–12 days after immunization with BCC However PE macrophages harvested from mice treated with T.A.B. or BCG showed no enhanced capacity to degrade complexes at this period. The state of immunity induced by BCG and T.A.B about 10 days after injection although not detected by a test for digestive activity does affect PE macrophages to the extent that they show an increased 1– ⁴C glucose oxidation (14) This suggests two possibilities a) proteolytic enzymes which degrade complexes in PE stimulated macrophages are not involved in bactericidal killing and b) the threshold for activating digestive capacity is greater than that required for activation of increased respiration (14)

In contrast to the results obtained with PE macrophages, cells taken from a focus of infection *i.e* BCG activated spleen cells—from C3H and C57Bl mice—degrade complexes to a greater extent than those from any of the other groups. Since BCG is injected i.v it multiplies in the spleen thus ensuring that antigen is continuously present for interaction with lymphocytes and macrophages resulting in the activation of the latter cells (12) This suggests that sufficient antigen and/or immunocompetent cells are not present in the peritoneum for the activation or direct stimulation of macrophages which would explain why PE macrophages from BCG immunized mice behave like normal cells. On the other hand, sufficient antigen must be present to cause the altered morphological appearance of the macrophages. There is some evidence

activated macrophages to suppress the intracellular growth of virulent *Listeria monocytogenes in vitro* as a means of distinguishing between these cells. The assay is based on the experiments of *Simon & Sheagren* (15) which were modified in order to facilitate comparisons between differently treated cell populations.

MATERIALS AND METHODS

Mice. C3H/Sec/1 of both sexes, CBA/Sec/1 male, bred at Statens Seruminstitut, and C57Bl/6J/BOM female purchased from Bomholtgård, Århus. All were 3–4 months old.

Bacteria. 2×10^3 *Listeria monocytogenes* (SSC 1423) were injected into the tail vein of each mouse 6 days prior to the experiments. A variant of this strain, which was rendered resistant to 16 μg/ml streptomycin, was used for infection of monolayers. LD_{50} for C3H mice for the streptomycin-sensitive strain is about 4×10^3 organisms and about 1×10^4 for the resistant organisms.

Approximately 10^7 viable units BCG were injected in 0.2 ml of Sauton medium into each mouse 13 days prior to the experiments.

Stimulants. 2 ml of a 10 per cent sterile solution of proteose-peptone (Difco) (PP) was injected intraperitoneally (i.p.) 3 days prior to cell harvest. Typhoid-paratyphoid vaccine (T.A.B.) was obtained from the Serum and Vaccine Department, Statens Seruminstitut. The vaccine was diluted tenfold in 0.9 per cent saline and 1 ml of this was injected i.p. 5 days prior to the experiments.

Preparation of macrophage monolayers. Peritoneal cells were harvested from 10–20 mice by washing out the peritoneum with 2.5 ml Minimal Essential Medium (MEM Eagle) containing 5 IU heparin/ml. The cells were pooled, counted and washed twice with MEM and adjusted to a concentration of 2.5×10^6 cells/ml MEM containing 20 per cent foetal calf serum, 0.4 per cent sodium bicarbonate, 50 μg/ml mycostatin and 5 μg/ml streptomycin = final culture medium (FCM). 2 ml of the cell suspension was placed in plastic Leighton tubes (*NUNCLON-Delta, 1409*) and these were incubated overnight in an atmosphere containing 5 per cent CO_2. In the experiments with cells from BCG immunized mice monolayers were prepared with or without the addition of PPD. The concentration of PPD was 2 μg/ml for cells from C3H and C57Bl mice, 2 μg/ml and 10 μg/ml for cells from CBA mice.

Infection of monolayers and determination of growth rate. 0.1 ml of listeria suspension, containing 1×10^6 listeria, was added directly to the culture tubes and control tubes containing culture medium but no cells, without prior washing of the monolayers. Cells and bacteria were in contact for 30 minutes in an atmosphere of CO_2 and subsequently the supernatants were removed and the number of bacteria in these was determined. The cell layer was washed once with 2 ml MEM and 2 ml FCM containing 3 MIC = 0.75 μg/ml penicillin was then added. The tubes were replaced in the incubator, this being the experimental zero time. After incubation for ½ hour or 4 hours, the supernatants were withdrawn from the tubes and the cells were lysed immediately by adding 2 ml distilled water and agitating vigorously in a Whirlimixer. The number of bacteria in the cell lysate was determined by means of a plating technique (see below).

Evaluation of results. In each experiment the number of bacteria in the lysed monolayers was estimated by means of a plating technique on blood agar plates at t = ½ hour and t = 4 hours. Each experiment was carried out in triplicate and the mean and standard deviation of the total count of each of these were calculated. The standard deviation was found to depend on the mean value, but was higher than the square root of this mean value indicating that the results could not be described by the Poisson distribution. Instead, it was inferred from the empirical data that the standard deviation could be estimated by multiplying the square root of the mean value by 2.2. The variance of the $\log_{10}$ mean value is therefore approximately $M^2 \times 4.84 \times \bar{X}/3 \times \bar{X}^2$ where $M = \log_{10}e = 0.43429$. The standard error of the $\log_{10}$ mean value is therefore $0.55/\sqrt{\bar{X}}$. This estimate was used for comparison of the generation times found in the different experiments. The generation time was estimated as follows. The mean value of the three determinations was multiplied by the corresponding dilution factor and the $\log_{10}$ value of this was determined = Y. The slope was calculated as $\frac{Y_4 - Y_{½}}{60\,(4 - ½)}$. The generation time was calculated as $\log_{10}2$/slope, assuming an exponential growth curve from t = ½ to t = 4 hours. Two generation times were compared by taking the difference between the corresponding slopes and dividing by the estimated standard error. Since the number of bacteria was counted at different dilutions (the mean values of bacteria ranged from 35 to 800) the standard error used also varies to a considerable extent.

RESULTS

Preliminary Studies on the Assay of the Generation Time of Listeria

Fig. 1 shows, schematically, the results of an experiment in which the listeria content

Acta path. microbiol. scand, Sect. C, 85 246-252, 1977

IN VITRO STUDIES ON NORMAL, STIMULATED AND IMMUNOLOGICALLY ACTIVATED MOUSE MACROPHAGES

III Intracellular Multiplication of Listeria monocytogenes

J Bennedsen, S Riisgaard J M Rhodes and S Olesen Larsen

Tuberculosis Department, WHO Collaborative Centre for Reference and Research on Escherichia, and Biostatistical Department, Statens Seruminstitut, Copenhagen, Denmark

Bennedsen J., Riisgaard, S Rhodes, J M & Olesen Larsen, S *In vitro* studies on normal, stimulated and immunologically activated mouse macrophages. III Intracellular multiplication of *Listeria monocytogenes* Acta path. microbiol. scand. Sect. C, 85 246-252 1977

The intracellular generation time of *Listeria monocytogenes* is increased in monolayers of PE macrophages from mice immunized with BCG listeria and T.A.B. vaccine as compared with monolayers of PE macrophages from normal mice stimulated intraperitoneally with proteose-peptone. Thus non-specifically stimulated and immunologically activated macrophages, although sharing several other characteristics, differ in their capacity to control intracellular bacterial infection.

Key words Macrophages activation *in vitro* mouse intracellular multiplication.

J Bennedsen Tuberculous Department, Statens Seruminstitut, Amager Boulevard 80 DK 2300 Copenhagen S Denmark.

Received 3.I.77 Accepted 3.II.77

In the two accompanying papers we have studied two aspects of a series of experiments concerned with the similarities and differences between immunologically activated and non specifically stimulated mouse macrophages (12, 14) The two assays described in these papers, *i e* determination of 1-^{14}C glucose oxidation and protein degradation emphasized the similarities between the two types of cells.

The next aspect of macrophage function to be analysed was its role in the control of infection It is a well known fact that the macrophage plays a dominant part in the fight against intracellular bacterial infections (7) Cell mediated immunity follows in the wake of such infections resulting in increased resistance to bacterial parasites. Macrophages obtained from loci of infection are altered morphologically and have a higher enzyme content than normal macrophages (4) Systemic macrophages in the peritoneum are also activated in the course of infection (8) Furthermore non-specific stimulation of the peritoneum with agents such as proteose-peptone (PP) causes an accumulation of macrophages in the peritoneal cavity which are morphologically similar to activated macrophages (11)

The present paper is concerned with studies on the ability of normal stimulated and

TABLE 1 *Intracellular Generation Time in Minutes of Listeria monocytogenes in Monolayer Culture of PE Macrophages*

Mice	Normal	PP	T.A.B.	BCG 0 µg/ml*	BCG 2 µg/ml	BCG 8 µg/ml*	L.m.
C3H	94	108	139†	111	121‖		119‖
CBA	92	97	130‡	287‡	486‡	199†	167†
C57Bl	123	92	161	136	251‖		164

* Concentration of PPD in FCM. ‖ $P<0.05$ † $P<0.01$ ‡ $P<0.001$
PP = Proteose-peptone, injected i.p.
T.A.B. = Typhus-paratyphus vaccine injected i.p
BCG = injected i.
L.m. = *Listeria monocytogenes* injected i.v

rate differed in a systematic way being most significantly expressed in the results obtained with CBA mice. The intracellular generation time in monolayers of normal cells showed a baseline of 92 minutes, and the same result was obtained with monolayers of cells from mice injected with PP 3 days before cell harvest. In contrast, i.p. injection of T.A.B. 3 days before cell harvest gave rise to a significantly increased doubling time ($P = 0.05$) the same result was obtained after immunization with BCG (13 days) and listeria (6 days) ($P = 0.001$ and 0.01). Specific antigen was not added to the latter cultures. A further increase in generation time was observed when monolayers of cells from mice immunized with BCG were prepared in FCM containing PPD but this increase was not significant. The same type of result was obtained with monolayers prepared from cells from C3H and C57Bl mice, but the values were less significant (see Table 1). It should be noted that the generation time in normal cells from C57Bl mice was rather high. In another series of experiments (not shown in the tables) it was found that the addition of PPD to monolayers prepared from the three strains of mice did not alter the growth rate. Bacterial counts were determined in all the initial supernatants in the experiments described here in order to measure initial killing by macrophages. A significant decrease in listeria concentration in the presence of macrophage monolayers was only observed in a few cases.

DISCUSSION

The macrophage monolayer culture infected with a facultative bacterial parasite represents a situation where extra- and intracellular microorganisms multiply at different rates and the parasites are phagocytosed and released from cells at different velocities. One single mathematical equation which covers all these events would be complicated and its parameters difficult to determine. Simplification of the system is, therefore, inevitable and this is usually attempted by minimizing the initial number of extracellular organisms by repeated washings and by controlling their multiplication with antibiotics.

In preliminary experiments, Fig. 1 representing a typical example, we found in accordance with the results of others (17) that it was impossible to eliminate extracellular listeria by repeated washings. We observed that the number of bacteria removed after each renewal of medium was related to the cell associated bacteria rather than to the content of organisms in the preceding supernatant. We suspect, therefore that each washing releases a certain proportion of the cell associated bacteria which in our experiments ranged from 0.2–0.4. Consequently the medium should only be removed when the extracellular bacteria exceed 0.2 × the intracellular content, which means that under our experimental conditions the cells should only be washed once, i.e. at the end of the incubation period.

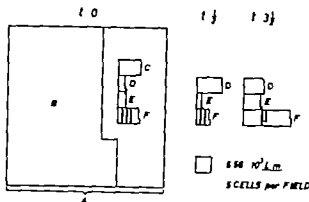


Fig 1 Number of *Listeria monocytogenes* found in various supernatants of the infected macrophage monolayers. Each supernatant is indicated by an area which is proportional to the number of organisms. Preliminary experiment monolayer + listeria → incubation 30 minutes → supernatant removed (B) → washing (C) → FCM added → incubation (0 ½ and 3½ hours) → FCM removed (D) → washing (E) → cell lysis in distilled water (F) A = number of listeria in control tubes without cells, removed simultaneously with B Number of cells per field refers to cells estimated microscopically after fixing and staining the monolayers.

was determined in the various supernatants removed from the monolayers after washing A significant portion (A B) of the listeria was removed from the extracellular compartment, irrespective of whether the organisms were killed or phagocytized. The subsequent washing fluid (C) contained about 2 per cent of the bacteria present in the supernatant (B) which is in accordance with an isotope dilution assay carried out on the residue remaining in the Leighton tubes.

When the FCM was added and then immediately removed (D) it contained about 33 per cent of the bacteria found in the preceding supernatant (C) The washing fluid (E) contained the same number of bacteria as in D The figure thus illustrates that apart from the initial washing the number of bacteria in each supernatant is related to the number of cell associated bacteria rather than to those present in the preceding supernatant.

Fig 1 also shows that the value for F at ½ hour was less than the zero value but that it increased after 3½ hours. The number of cells per field decreased from 0 to ½ hour but was then stable up to 3½ hours as determined microscopically

The effect of various concentrations of penicillin in the culture medium on the intracellular growth of *Listeria monocytogenes* is shown in Fig 2 The minimal inhibitory concentration of penicillin in FCM on extracellular growth was estimated to be 0.25 μg/ml There was no effect of variation in the dose of penicillin (2–16 MIC) on intracellular growth after 4 hours incubation, regardless of whether the macrophages were obtained from normal or BCG immunized mice. However intracellular growth was reduced after the addition of 32 MIC penicillin to cultures of normal cells.

Intracellular Generation Times of Listeria in Monolayers of PE Macrophages from Normal Stimulated and Activated Mice

Table 1 illustrates the generation times obtained in the various monolayers. These generation times were calculated from the bacterial counts found at ½ and 4 hours incubation multiplied by the corresponding dilution factor (see Material and Methods, evaluation of results)

The table shows that a positive growth was obtained in all cases, but that the growth

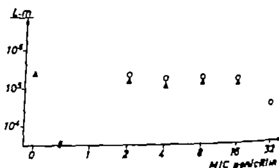


Fig 2 The effect of extracellular penicillin on the intracellular concentration of listeria in monolayers of PE macrophages from normal mice experiment 1 (●) and experiment 2 (○) and BCG immunized mice experiment 3 (▲) after incubation at 37 C for 4 hours. MIC = 0.25 μg/ml penicillin. 10 μg/ml PPD was added to the culture medium in experiment 3

TABLE 1 *Intracellular Generation Time in Minutes of* Listeria monocytogenes *in Monolayer Culture of PE Macrophages*

Mice	Normal	PP	T.A.B.	BCG 0 µg/ml*	BCG 2 µg/ml	BCG 8 µg/ml*	L.m.
C3H	94	108	139†	111	121§		119§
CBA	92	97	130§	287‡	406‡	199†	167†
C57Bl	123	92	161	136	251§		164

* Concentration of PPD in FCM, § $P<0.05$ † $P<0.01$ ‡ $P<0.001$
PP = Proteose-peptone injected i.p.
T.A.B. = Typhus-paratyphus vaccine injected i.p.
BCG = injected i.v.
L.m. = *Listeria monocytogenes* injected i.v.

rate differed in a systematic way being most significantly expressed in the results obtained with CBA mice. The intracellular generation time in monolayers of normal cells showed a baseline of 92 minutes, and the same result was obtained with monolayers of cells from mice injected with PP 3 days before cell har vest. In contrast, i.p. injection of T.A.B. 5 days before cell harvest gave rise to a significantly increased doubling time ($P = 0.05$) the same result was obtained after immunization with BCG (13 days) and listeria (6 days) ($P = 0.001$ and 0.01). Specific antigen was not added to the latter cultures. A further increase in generation time was observed when monolayers of cells from mice immunized with BCG were prepared in FCM containing PPD but this increase was not significant. The same type of result was obtained with monolayers prepared from cells from C3H and C57Bl mice, but the values were less significant (see Table 1). It should be noted that the generation time in normal cells from C57Bl mice was rather high. In another series of experiments (not shown in the tables) it was found that the addition of PPD to monolayers prepared from the three strains of mice did not alter the growth rate. Bacterial counts were determined in all the initial supernatants in the experiments described here in order to measure initial killing by macrophages. A significant decrease in listeria concentration in the presence of macrophage monolayers was only observed in a few cases.

DISCUSSION

The macrophage monolayer culture infected with a facultative bacterial parasite represents a situation where extra and intracellular microorganisms multiply at different rates and the parasites are phagocytized and rele ased from cells at different velocities. One single mathemathical equation which covers all these events would be complicated and its parameters difficult to determine. Simplification of the system is, therefore, inevitable and this is usually attempted by minimizing the initial number of extracellular organisms by repeated washings and by controlling their multiplication with antibiotics.

In preliminary experiments, Fig. 1 representing a typical example, we found in accordance with the results of others (17) that it was impossible to eliminate extracellular listeria by repeated washings. We observed that the number of bacteria removed after each renewal of medium was related to the cell associated bacteria rather than to the content of organisms in the preceding supernatant. We suspect, therefore, that each washing releases a certain proportion of the cell associated bacteria which in our experiments ranged from 0.2–0.4. Consequently the medium should only be removed when the extracellular bacteria exceed 0.2 × the intracellular content, which means that under our experimental conditions the cells should only be washed once i.e. at the end of the incubation period.

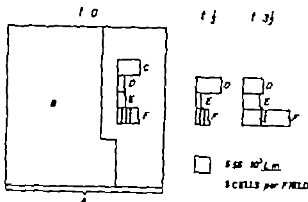


Fig 1 Number of *Listeria monocytogenes* found in various supernatants of the infected macrophage monolayers. Each supernatant is indicated by an area which is proportional to the number of organisms. Preliminary experiment monolayer + listeria → incubation 30 minutes → supernatant removed (B) → washing (C) → FCM added → incubation (0 ½ and 3½ hours) → FCM removed (D) → washing (E) → cell lysis in distilled water (F) A = number of listeria in control tubes without cells, removed simultaneously with B Number of cells per field refers to cells estimated microscopically after fixing and staining the monolayers.

was determined in the various supernatants removed from the monolayers after washing A significant portion (A B) of the listeria was removed from the extracellular compartment, irrespective of whether the organisms were killed or phagocytized. The subsequent washing fluid (C) contained about 2 per cent of the bacteria present in the supernatant (B) which is in accordance with an isotope dilution assay carried out on the residue remaining in the Leighton tubes.

When the FCM was added and then immediately removed (D) it contained about 33 per cent of the bacteria found in the preceding supernatant (C) The washing fluid (E) contained the same number of bacteria as in D The figure thus illustrates that apart from the initial washing the number of bacteria in each supernatant is related to the number of cell associated bacteria rather than to those present in the preceding supernatant.

Fig 1 also shows that the value for F at ½ hour was less than the zero value, but that it increased after 3½ hours. The number of cells per field decreased from 0 to ½ hour but was then stable up to 3½ hours as determined microscopically

The effect of various concentrations of penicillin in the culture medium on the intracellular growth of *Listeria monocytogenes* is shown in Fig 2 The minimal inhibitory concentration of penicillin in FCM on extracellular growth was estimated to be 0.25 µg/ml There was no effect of variation in the dose of penicillin (2–16 MIC) on intracellular growth after 4 hours incubation, regardless of whether the macrophages were obtained from normal or BCG immunized mice However intracellular growth was reduced after the addition of 32 MIC penicillin to cultures of normal cells.

Intracellular Generation Times of Listeria in Monolayers of PE Macrophages from Normal Stimulated and Activated Mice

Table 1 illustrates the generation times obtained in the various monolayers. These generation times were calculated from the bacterial counts found at ½ and 4 hours incubation multiplied by the corresponding dilution factor (see Material and Methods, evaluation of results)

The table shows that a positive growth was obtained in all cases, but that the growth

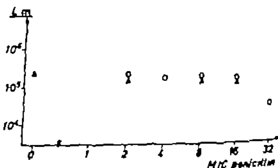


Fig 2 The effect of extracellular penicillin on the intracellular concentration of listeria in monolayers of PE macrophages from normal mice experiment 1 (●) and experiment 2 (○) and BCG immunized mice experiment 3 (▲) after incubation at 37 C for 4 hours. MIC = 0 25 µg/ml penicillin. 10 µg/ml PPD was added to the culture medium in experiment 3

is highly significant in monolayers from CBA mice, and a significant increase was also observed in cells from listeria immunized mice. However the increase in generation time observed with cells from C57Bl mice was not significant, which is probably due to the unusually high value of the normal control. The addition of antigen did not cause a significant increase in the generation time in any of the cells from the three strains of mice immunized with BCG. There is, therefore evidence that systemic macrophages, found in the peritoneum in the course of a BCG or listeria infection are activated *in vivo* and that this activation can be demonstrated by measuring the inhibition of intracellular growth *in vitro*.

We have shown in the accompanying papers (12, 14) that both PP stimulated and BCG activated PE macrophages showed an increased 1-^{14}C glucose oxidation, whereas the former cells had a higher digestive capacity than the latter. On the other hand, BCG activated PE macrophages degraded complexes to the same extent as normal cells. Determination of the generation time could distinguish between PP stimulated and BCG activated macrophages, since in this assay PP cells resembled normal cells. A summary of the results found with the three assays is shown in Table 2. It should be noted that two of the three assays (see Table 2) were carried out with monolayer cultures, whereas cell suspensions were used for the studies on digestive capacity. Degradation of complexes in monolayers, although following the same pattern observed with cell suspensions (own unpublished observations) is very low and, therefore cell suspensions were used in order to obtain more meaningful results. A discussion of the reasons why BCG activated PE macrophages are not capable of degrading complexes better than normal cells is given in article number 2 (12).

It should also be borne in mind that although BCG activated PE macrophages do not digest complexes better than normal macrophages, spleen macrophages (from a focus of infection) do so to a greater extent than any of the other groups of cells, including normal cells (12). Thus, activated spleen macrophages degrade complexes efficiently and they show an increased 1-^{14}C glucose oxidation, but we have not investigated whether they inhibit intracellular growth of listeria.

T.A.B. vaccine yielded some interesting results which were a combination of those obtained with PP and BCG. PE macrophages from T.A.B. vaccinated mice (5 days, see Table 2) showed increased 1-^{14}C glucose oxidation digestive capacity and inhibition of growth. PP stimulated macrophages showed no inhibition of growth but the two other parameters were increased, whereas BCG macrophages showed increased oxidation and inhibition of growth but not digestive capacity. There are two possible explanations for the effect of T.A.B. vaccine in our assays: a) it gives rise to cell mediated immunity and in this way produces immunological activation of macrophages, hence the increase in generation time of listeria *in vitro* in PE macrophages. We have also found that the injection of T.A.B. vaccine i.p. gives rise to non-specific resistance to listeria *in vivo* (15). b) it also acts as a non-specific stimulant which is as effective as PP stimulation with regard to increase in respiration and protein degradation.

It is of interest that *Reikvam et al.* (10) observed a decrease in the number of intracellular listeria in macrophages from mice immunized with *Toxoplasma gondii*, whereas they found an increase in the number of listeria in macrophages which were stimulated *in vitro* in newborn calf serum. In other respects, the two cell types resembled each other. Growth inhibition studies of listeria in suspension cultures were carried out by *Ratzan et al.* (9) who found enhanced inhibition in the presence of PE macrophages from BCG or listeria infected animals, but not from normal animals. This inhibition correlated with an increase in 1-^{14}C glucose oxidation, which agrees with our results.

The overall conclusion arising from the study of the three assay systems described in

TABLE 2. *Summary of Results Obtained with the 1–¹⁴C Oxidation Assay (14) Protein Degradation Assay (12) and Intracellular Generation Time Assay (this Report)*

Injected with	Cell harvest on day	PE macrophages			Spleen macrophages		
		1–¹⁴C-oxidation*	Degradation§	Generation time*	1–¹⁴C-oxidation*	Degradation§	Generation time*
O		—	—	—	—	—	n.d.
PP	3	+	+	—	—	—	n.d.
T.A.B	5	+	+	+	n.d.	—	n.d.
BCG	12–13	+	—	+	+	+	n.d.
L.m.	6	+	—†	+	n.d.	—	n.d.

n.d. = not done
* = results from monolayers.
§ = results from cell suspensions.
† = results not included in article
\+ = enhancement.
— = background value.
For explanation of abbreviations, see Table I.

The decrease in intracellular listeria from t = 0 to t = ½ hour, which was a regular feature of these experiments, is correlated to the decrease in cell density during the same time interval and might feasibly be explained by a release of partly damaged macrophages arising during the many manipulations after phagocytosis. In order to circumvent this difficulty we measured the number of extra cellular bacteria after ½ and 4 hours and expressed the results as the generation time based on these two determinations.

Since a significant proportion of the cell associated microorganisms will be found ex tracellularly after addition of FCM to the monolayers and the extracellular growth rate is higher than the intracellular growth rate subsequent phagocytosis will contribute con siderably to the intracellular content If extra cellular growth can be controlled its effect on the calculation of the generation time will be of little significance The addition of anti biotics to the extracellular environment has been criticized (5) and it is only justifiable if the antibiotics do not exert an intracellular effect.

We found that variation in the penicillin content from 2–16 MIC (0 5–4 μg/ml) had no effect on the number of intracellular mi-croorganisms (Fig 2) This finding only partly agrees with that of *Cole* (2) who ob-served that more than 1.2 μg/ml penicillin in-hibited the intracellular growth of listeria, after 18 hours. *Bonventre* & *Imhoff* (1) have reported that streptomycin accumulated with-in macrophages as a result of active trans-port If this observation can be applied to penicillin a smaller proportion of the extra-cellular concentration of the antibiotic would be transported into the cells at 4 hours than after 18 hours, which would explain the dis-crepancy between our results and those of *Cole*

Table I shows that under the experimental conditions described under Materials and Methods listeria is able to multiply inside monolayers of normal peritoneal macrophages *in vitro* with a generation time of 90–95 minutes. This multiplication is inhibited in cells from BCG immunized mice when the culture medium contains specific antigen. A similar observation was described by *Simon* & *Sheagren* (15) who used oil induced guinea pig macrophages. These findings can be explained by the presence of lymphocytes in the exudate which, in the presence of anti-gen are responsible for the *in vitro* activation of macrophages (3 6 16) However we found that when specific antigen was not added to the monolayers of PE macrophages, obtained from BCG and listeria immunized mice, there was some increase in generation time when compared with the value obtained with cells from normal mice. This increase

Acta path. microbiol. scand. Sect. C, 85 253–259 1977

AN *IN VITRO* ASSAY FOR THE QUANTITATION OF PHAGOCYTIC CELLS OF DIFFERENT ANATOMIC ORIGIN

BIRGITT HERTEL WULFF

Immunobiology Laboratory, Statens Seruminstitut, Copenhagen, Denmark

Hertel-Wulff B. An *in vitro* assay for the quantitation of phagocytic cells of different anatomic origin. Acta path. microbiol. scand. Sect. C 85 253–259 1977

The survival of peritoneal exudate macrophages after 3 to 10 days in culture was examined by measuring the numbers of phagocytes per culture. This was determined by letting the cultured cells phagocytize Latex particles. The number of Latex particle-containing cells was taken as measure of the survival of phagocytes. It was found that one tenth of the cells judged by light microscopy as macrophage-like survived the culture period. Thus, the calculated plating factor of 9.3 was used to estimate the actual number of macrophages in suspensions of spleen, lymph node or thymus cells by culturing these cells and subsequently counting Latex particle-containing cells. In addition, the acridine orange technique was used to determine actual numbers of macrophages in freshly prepared cell suspensions of lymphoid organs. Latex studies on spleen and thymus cells gave results correlating well with data obtained by the acridine orange technique. By contrast, many more acridine orange positive cells than phagocytizing cells were found when lymph node cells were cultured.

Key words: Phagocytic cells; different anatomic origin; quantitation; *in vitro*.

B. Hertel-Wulff, Immunobiology Laboratory, Blood Grouping Department, Statens Seruminstitut, Amager Boulevard 80, DK-2300 Copenhagen S, Denmark.

Received 12.x.76 Accepted 24.ii.77

In studies on cellular requirements for lymphocyte proliferation *in vitro* against hapten-carrier conjugates or proteins (5) or for induction of cytotoxic T lymphocytes *in vitro* (6) use has been made of a great variety of responding or stimulating cell mixtures. A special interest has focused on the role of macrophages in the inductive phase of these cellular processes, i.e. on their cooperation with other immunologically important cell types. The effect of freshly harvested as well as of cultured macrophages has been studied (6).

This paper deals with the study of simple methods to obtain knowledge of the survival of macrophages cultured for several days. The identification and quantitation of macrophages was performed by their ability to phagocytize as well as by their high content of lysosomal granules.

The cells for this study were obtained from mice 5–7 days after an intraperitoneal injection of incomplete Freund's adjuvant (IFA) and thus, this material mainly consisted of stimulated macrophages. Light, phase and electron microscopic appearances of stimulated macrophages resembles those of other macrophages except that the stimulated cells contain large numbers of granules in their cytoplasm and large irregularly shaped vacu-

these three articles must be that the determination of the intracellular multiplication is the only assay in our hands which can conclusively distinguish *in vitro* between PE macrophages activated and stimulated *in vivo*. This again implies that although respiration and digestive capacity (*i.e.* content of proteolytic enzymes) appear to be similar in both types of cells, some bactericidal mechanism is present in activated cells which does not appear to be present to the same degree in stimulated cells. This difference may of course be more apparent than real since it is possible that any difference may be quantitative rather than qualitative.

The mechanism whereby macrophages are stimulated non-specifically to attain a similar but not necessarily identical state to the activated macrophages is still obscure. This point requires further elucidation in order that the macrophage can be fully exploited in the fight against infection and neoplasia.

We would like to acknowledge the expert secretarial assistance of *A. Thorborg*, the excellent technical assistance of *Ulla Bjørn Rasmussen* and *A. G. Overgaard* for the drawings.

REFERENCES

1. *Bonventre P. F. & Imhoff J. G.*: Uptake of ³H-dihydrostreptomycin by macrophages in culture. Infect. Immun. 2 89–95 1970.
2. *Cole P.*: Intracellular killing of *Listeria monocytogenes* by activated macrophages (Mackaness system) is due to antibiotic. Nature (Lond.) *256* 515–517 1975.
3. *Cole P.*: Activation of mouse peritoneal cells to kill *Listeria monocytogenes* by T-lymphocyte products. Infect. Immunol. *12* 36–41 1975.
4. *Dannenberg A. M. Meyer O. T. Esterly J. R. & Kambara T.*: The local nature of immunity in tuberculosis, illustrated histochemically in dermal BCG lesions. J. Immunol. *100* 931–941 1968.
5. *Hart P. D'Arcy*: Critical approach to the technique of assessment of antibacterial effects of activated mouse peritoneal macrophages. In *Wagner W. H. & Hahn H.*: Activation of Macrophages. Excerpta Medica, Amsterdam 1974 p. 131–137.
6. *Krahlenbuhl J. L. Rosenberg, L. T. & Remington J. S.*: The role of thymus-derived lymphocytes in the *in vitro* activation of macrophages to kill *Listeria monocytogenes*. J Immunol *111* 992–995 1973.
7. *Mackaness G. B. & Blanden R. V.*: Cellular immunity. In *Mudd S.* (Ed.) Infectious Agents and Host Reactions. W. B. Saunders Co. Philadelphia 1970 p. 22–60.
8. *North R. J.*: Cellular kinetics associated with the development of acquired cellular resistance. J. exp. Med. *130* 299–311 1969.
9. *Ratzan K. R., Musher D. M., Keusch, G. T. & Weinstein L.*: Correlation of increased metabolic activity, resistance to infection, enhanced phagocytosis, and inhibition of bacterial growth by macrophages from listeria- and BCG-infected mice. Infect. Immun. *5* 499–504 1972.
10. *Reikvam A. Grammeltvedt R. & Høiby E. A.*: Activated mouse macrophages: morphology, lysosomal biochemistry and microbicidal properties of *in vivo* and *in vitro* activated cells. Acta path. microbiol. scand. Sect. C, *83* 129–138, 1975.
11. *Rhodes J. M.*: *In vitro* studies on the fate of antigen. 5. The uptake of I¹³¹ HSA by mouse exudate cells in the presence of normal serum and anti-HSA serum. Acta path. microbiol. scand. 65 457–466 1965.
12. *Rhodes J. M. Nielsen G., Olesen Larsen S. Bennedsen J. & Riisgaard S.*: *In vitro* studies on normal, stimulated and immunologically activated mouse macrophages. II. Degradation of radioactive antigen/antibody complexes. Acta path. microbiol. scand. Sect. C, *85* 239–245 1977.
13. *Rhodes J. M. Bennedsen J. & Olesen Larsen S.*: Unpublished data.
14. *Riisgaard S. Bennedsen J. & Rhodes J. M.*: *In vitro* studies on normal, stimulated and immunologically activated mouse macrophages. I. Oxidation of 1-¹⁴C glucose by macrophages in monolayer cultures. Acta path. microbiol. scand. Sect. C *85* 233–238, 1977.
15. *Simon H. B. & Sheagren J. N.*: Cellular immunity *in vitro*. I. Immunologically mediated enhancement of macrophage bactericidal capacity. J. exp. Med. *133* 1377–1389 1971.
16. *Simon H. B. & Sheagren J. N.*: Enhancement of macrophage bactericidal capacity by antigenically stimulated immune lymphocytes. Cell Immunol. *4* 163–174 1972.
17. *Wilder M. S. & Edberg J. C.*: Interaction of virulent and avirulent *Listeria monocytogenes* with cultured mouse peritoneal macrophages. Infect. Immun. *7* 409–415 1973.

TABLE 1. *Numbers of Phagocytic Cells among CBA PE Macrophages Determined by Latex Particle Uptake after 3 5 7 & 10 Days in Culture*

Days in culture	Mean of triplicate	Numbers of PE macrophages plated per well[a)]				
		5×10^4	1×10^4	2×10^3	4×10^3	8×10^2
3	No. of LCC[b)] per well	Too many	3 412	1,050	170	24
	Per cent LCC[c)]		3 4	5.2	4.2	3.0
5	No. of LCC per well	3 798	5,758	1,076	310	38
	Per cent LCC	0 8	5.8	5.4	7.8	4.8
7	No. of LCC per well	4 140	8,236	1,071	236	6
	Per cent LCC	0.8	8.2	5.4	5.9	0.8
10	No of LCC per well	3,070	8,400	2,970	575	10
	Per cent LCC	0.6	8 4	14.8	14.4	1.3

a) Judged as seen in the light microscope (magnification = 400 ×) 77.6 per cent of PEC stained with acridine orange, 61.5 per cent of PEC were EAC-RFC and 42.6 per cent of PEC were EA-RFC.

b) LCC = Latex particle containing cells.

c) Per cent LCC = $\frac{\text{mean numbers of LCC per well}}{\text{number of PE macrophages plated}} \times 100$

RESULTS

Latex digestion by PEC

CBA mice gave peritoneal exudate cells (PEC) containing 10.1 per cent of lymphocytes estimated under the light microscope. 5×10^3 1×10^3 2×10^4 4×10^3 or 8×10^2 PE macrophages were cultured in microtiter plates in triplicates for 3, 5 7 or 10 days and on the day in question tested for the ability to take up Latex particles. Table 1 shows the number of Latex particle containing cells (LCC) and the per cent of phagocytic cells related to the number of plated cells judged as seen in the light microscope (one representative experiment) On days 3 and 5 there was a good correlation between per cent phagocytic cells seen in PEC cultures containing 8×10^2–1×10^3 macrophages at the start of the culture period but after 5 days in culture lower numbers of macrophages plated ($<4\times10$ per well) resulted in ery low recovery Furthermore the day 3 cultures contained many fibroblast-like cells and lymphocytes, while day 5 7 or 10 cultures contained very few lymphocytes and many fibroblast-like cells. The fibroblast like cells some of which phagocytized Latex particles could be macrophages which put out pseudopodia and they were included as LCC.

This spreading phenomenon has been seen among macrophages in culture (8) and is thought to be an example of phagocytic ac tivity where the cells attemp to phagocytize infinitely large particles (8)

Plating 4×10^3–1×10^4 cells per well the numbers of LCC increased following culture. Whether this was due to division of macrophages (11) macrophage recrutement from fibroplasts or from lymphocyte-like cells was impossible to say

Granule size and hydrolase activity have been reported to increase when mouse peritoneal phagocytes were cultured in medium containing 50 per cent FCS instead of 20 per cent FCS (2) In the present study no influence of the FCS concentration (increased from 10 per cent to 25 per cent) upon the survival of phagocytic cells determined by Latex particle ingestion was seen.

Table 2 shows the mean plating efficiency as tested by Latex particle uptake in 10 ex periments counted 3 days after culture and in

oles (3) Although the cells of the mononuclear phagocytic family present a wide range of structural features, they all adhere to glass and plastic surfaces, have ruffled membranes and engage in pinocytosis as well as phagocytosis. Consequently the survival of macrophages was examined after 3, 5 7 or 10 days in culture by measuring the numbers of phagocytizing cells per culture. This was compared to the number of macrophages in a freshly harvested suspension of peritoneal exudate cells (PEC) determined by the acridine orange technique which stains lysosomes red.

MATERIALS AND METHODS

Animals In the present study 8–13 weeks old CBA, Balb/C and Balb/C×DBA mice were used.

Preparation of macrophages Tissue culture medium RPMI 1640 (Gibco N Y) supplemented with penicillin (100 I U./ml) glutamine (2 mM) gentamicin (10 μg/ml) HEPES (N 2 hydroxyethylpiperazine N 2-ethane sulfonic acid, 10 mM and 10 per cent fetal calf serum (FCS) (Gibco lot. no C553018) was used throughout these studies. Peritoneal exudate cells (PEC) were harvested from mice, injected i.p 5–7 days previously with 0.5 ml of buffered salt solution (BSS) emulsified in incomplete Freund's adjuvant (IFA) by flushing out the peritoneal cavity with 2 × 5 ml of sterile medium containing 50 I U of heparin (Lovens kemiske Fabrik, Copenhagen) per ml. Cells were washed 3 times in medium and the number of viable cells larger than lymphocytes as well as the number of lymphocytes were determined by the trypan blue method (9) The number of macrophages determined in this way was between 15.2×10^{6} and 39.5×10^{6} per mouse (average $= 19.5\times10^{6} \pm 7.8\times10^{6}$) depending upon the time of harvest after i p stimulation, and between 10 1 per cent and 27 4 per cent of the PEC were lymphocytes (average = 16 8 per cent ± 4.9 per cent)

Culture of PEC Different numbers of PEC ranging from 8×10^{3} to 1×10^{5} cells were cultured in 0.2 ml of medium in triplicate using flat-bottom Micro Test II Tissue Culture Plates (no 3040 Falcon Plastics, 1950 Williams Drive, Oxnard Ca. 93030 USA) for 3 days or for 7 days at 37 C/5 per cent CO_2.

Cell suspension from spleen lymph node or thymus were made aseptically the cells being washed twice in medium. The number of trypan blue excluding cells was determined and different numbers of spleen cells (ranging from 3×10^{5} to 1×10^{6}) of lymph node cells (ranging from 1×10^{6} to 3×10^{6}) or of thymus cells (ranging from 5×10^{6} to 10×10^{6}) were cultured in 0.2 ml of medium in triplicate using microtiter plates for 3 and for 7 days in 37 C/5 per cent CO_2.

Characterization of macrophages On the day of PEC harvest macrophages were identified by means of the acridine orange (AO) technique (4) A 0.01 per cent (w/v) solution of acridine orange (Fluka AG Switzerland) was made in 10 per cent FCS/F15 Eagle's medium. This stock solution was kept at 4 C protected from light. 5×10^{4} cells were resuspended in 1 ml AO diluted 1:10 prior to use. The mixture was incubated for 30 minutes at 37° C and the cells washed once in 10 per cent FCS/F15 before counting under the UV light microscope (magnification 400 ×) Macrophages were defined as lymphoid cells with a green nucleus and large cytoplasm containing 6 or more red coloured spots (lysosomes) (4)

Latex particle ingestion by phagocytic cells on day 3 or day 7 in culture was determined using Bacto-Latex 0.81 particles (Difco Laboratories) 0 1 ml of the Latex solution was added to 9.9 ml of 10 per cent FCS/F15 and centrifuged for 15 minutes at 2,000 × g at 4 C in order to remove larger aggregates. The medium in the microcultures of PEC was sucked away 0 1 ml of the Latex solution was added and the mixture was incubated for one hour at 37 C/5 per cent CO_2. The cells were then washed twice and 0 1 ml of medium added before identifying cells which had ingested 10 or more particles under the light microscope (magnification 320 ×)

Rosette assays The numbers of EA-rosette forming macrophages (E = sheep erythrocytes (SRBC) A = mouse 7S anti-SRBC antibody) and of EAC-rosette forming macrophages (E = SRBC, A = rabbit 19S anti-SRBC antibody C = C5 deficient mouse serum) were determined as described previously (1 9) A rosette forming macrophage was defined as a large cell having three or more target erythrocytes bound to its surface.

Antiserum Anti-θ antiserum (AKR anti-C3H thymocyte antiserum) was prepared and the cytotoxicity test performed as described previously (10)

Statistics The geometric mean and SD (standard deviation) of the numbers of LCC (Latex containing cells) in triplicate culture wells were calculated. The numbers will be given as per cent LCC = mean number of LCC per well multiplied by 100 and divided by the number of PE macrophages plated. 1.3 per cent ≦ SD ≦ 36.5 per cent of the mean number of LCC per well. The SD was higher the fewer PE macrophages were plated per well. By linear regression of the paired data ((x,y) = (the number of PE macrophages plated, the mean number of LCC per well)) the slope b, of the straight line obtained was calculated and the per cent survival of phagocytes determined.

TABLE 4 *Per Cent of Macrophage-like Cells in PEC from Different Mouse Strains which Stain with Acridine Orange (AO) and which Form EAC- or EA-R settes*

Mouse strain	Per cent macrophage-like cells[a]	Per cent AO cells[b]	Per cent EAC-RFC	Per cent EA-RFC
CBA	88.8	77.6	61.5	42.6
	74.5	–	–	25.4
	85.4	61.0	30.4	46.2
	77.8	54.4	36.4	22.5
	88.7	–	–	–
	87.5	67.9	62.0	29.1
	87.7	88.5	15.9	–
Average	84.3 ± 5.8	69.9 ± 13.5	41.2 ± 20.2	33.2 ± 20.6
Balb/C×DBA	72.6	–	–	–
	86.7	68.4	33.8	30.5
	84.3	–	–	–
	77.4	–	–	–
	84.8	–	–	–
	83.6	62.5	–	–
	83.9	–	–	–
	78.7	–	–	–
	87.3	89.3	19.8	–
Average	82.1 ± 4.9	73.4 ± 14.1	26.8 ± 9.9	30.5
Balb/C	80.5	58.5	13.7	–
	87.1	78.6	21.5	–
Average	83.7 ± 4.8	68.6 ± 14.2	17.6 ± 5.5	–
Average of total	83.2 ± 5.0	70.7 ± 12.3	32.8 ± 18.2	36.1 ± 11.8

a) Judged as seen in the light microscope (magnification = 400 ×)

b) Lymphoid cells with green nucleus and large cytoplasma containing 6 or more red coloured spots (lysosomes)

8 per cent lower (average = 12.7 ± 3.5 per cent) than the number of macrophage-like cells determined using the light microscope. This light microscope method was unable to discriminate macrophages and polymorphonuclear leucocytes of the same size, but by the AO technique it was possible to distinguish between these two cell types. This might explain the lower counts of AO cells. The rosette data differ very much from the AO cell counts, and it was concluded that of the four techniques the AO technique may give the most exact reflection of the actual number of macrophages in a freshly prepared cell suspension.

Estimation of the Numbers of Macrophages in Mouse Spleen, Lymph Node or Thymus

Between 3×10^4 and 1×10^6 Balb/C spleen cells, 1×10^5 and 3×10^6 lymp node cells, or 3×10^6 and 10×10^6 thymus cells were plated in triplicate cultures. When counting LCC in day 3 cultures of cells from the three lymphoid organs no fibroblast like cells were seen, whereas the day 7 cultures contained many of these cells. Table 5 shows the results of LCC counting and AO staining, respectively in one representative experiment. The calculated mean number of phagocytic cells among freshly prepared spleen cells (Table 5) was 3.49 per cent and among freshly prepared

TABLE 2. *The Mean per Cent of LCC*[a] *after 3 or 7 Days in Culture of PE Macrophages*

Days in culture	Numbers of PE macrophages plated per well[b]					
	1×10^5	2×10^4	6.7×10^3	4×10^3	2.2×10^3	8×10^2
3	5.0	8.9	9.7	6.1	9.8	3.9
7	3.8	6.3	12.2	2.8	9.5	1.3

a) See Table 1 b) and c)

b) Judged as seen in the light microscope (magnification = 400 ×)

7 experiments counted 7 days after culture. The data obtained from the day 3 cultures were similar when between 2.2×10^3 and 2×10^4 PE macrophages were plated whereas the plating efficiency when more than 2×10^4 (1×10^5) or less than 2.2×10^3 (8×10^2) macrophages were plated was somewhat lower. The same tendency was seen among the data from day 7 cultures although the results were more variable. Thus it seemed that an exact reflection of the survival of macrophages in culture required plating more than 2×10^3 and less than 2×10^4 macrophage-like cells per well in the culture system used here. Linear regression analysis of the paired data from the day 3 cultures excluding the per cent LCC obtained by plating 8×10^2 or 1×10^5 PE macrophages, gave a straight line. Calculating the slope s gave $s = 0.1285$. The corresponding results from the day 7 cultures gave $s = 0.0859$. It was concluded that plating between 2×10^3 and 2×10^4 PE macrophages resulted in a survival of 10.7 per cent, and thus, in a plating factor of $100/10.7 = 9.3$.

Effect of Anti-Θ Antiserum and Complement on the Survival of Phagocytes

Attempts at studying the effect of autologous macrophages on the generation of cytotoxic T lymphocytes (6) required knowledge of the *in vitro* behavior of anti-Θ antiserum plus complement treated macrophages. Theta positive depleted PEC were grown *in vitro* and their phagocytizing ability examined after different culture periods. Table 3 shows the per cent of phagocytic cells related to the number of plated cells before or after treatment with anti-Θ antiserum and complement in one out of three experiments. This treatment did not affect the survival of phagocytic cells.

Acridine Orange Staining of PEC

Table 4 shows the per cent of macrophage-like cells in PEC from three different mouse strains as determined by acridine orange (the AO cells). The number of AO cells from CBA, Balb/C × DBA or Balb/C mice was very similar and in all three cases more than

TABLE 3. *Per Cent LCC among Anti-Θ Antiserum and Complement Treated Balb/C PE Macrophages after 3 or 7 Days in Culture*

Days in culture	Anti-Θ + C[a]	Numbers of PE macrophages plated per well[b]			
		2×10^4	6.7×10^3	2.2×10^3	7.4×10^2
3	−	5.67	8.14	8.28	6.74
	+	6.63	6.55	5.15	4.04
7	−	11.67	8.43	13.09	7.33
		16.44	8.62	7.08	2.65

a) − = cells treated with complement (per cent AO cells 78.6 per cent per cent EAC-rosette forming macrophages 21.5 per cent) + = cells treated with anti-Θ antiserum plus complement (per cent AO cells 83.0 per cent per cent EAC-rosette forming macrophages 25.0 per cent)

b) Judged as seen in the light microscope (magnification = 400 ×)

TABLE 4. *Per Cent of Macr phage-like Cells in PEC from Different Mouse St ains which Stain with Acridine Orange (AO) and whi h F rm EAC- or EA-R settes*

Mouse strain	Per cent macrophage-like cells[a]	Per cent AO cells[b]	Per cent EAC-RFC	Per cent EA RFC
CBA	88.8	77.6	61.5	42.6
	74.5	–	–	25 4
	85 4	61 0	30.4	46.2
	77.8	54 4	36 4	22.5
	88.7	–	–	–
	87.5	67.9	62.0	29 1
	87.7	88 5	15.9	–
Average	84.3 ± 5.8	69.9 ± 13.5	41.2 ± 20 2	33.2 ± 20.6
Balb/C×DBA	72.6	–	–	–
	86 7	68.4	33.8	50.5
	84.5	–	–	–
	77 4	–	–	–
	84.8	–	–	–
	83.6	62.5	–	–
	83.9	–	–	–
	78 7	–	–	–
	87.3	89.3	19 8	–
Average	82.1 ± 4.9	73.4 ± 14 1	26.8 ± 9.9	50.5
Balb/C	80.3	58.5	13.7	–
	87 1	78 6	21.5	–
A erage	83 7 ± 4.8	68.6 ± 14.2	17.6 ± 5.5	–
A erage of total	83.2 ± 5.0	70.7 ± 12.3	32.8 ± 18.2	36.1 ± 11.8

a) Judged as seen in the light microscope (magnification = 400 ×)

b) Lymphoid cells with green nucleus and large cytoplasma containing 6 or more red coloured spots (lysosomes)

8 per cent lower (average = 12.7 ± 3.5 per cent) than the number of marcrophage-like cells determined using the light microscope. This light microscope method was unable to discriminate macrophages and polymorphonuclear leucocytes of the same size but by the AO technique it was possible to distinguish between these two cell types. This might explain the lower counts of AO cells. The rosette data differ very much from the AO cell counts, and it was concluded that of the four techniques the AO technique may give the most exact reflection of the actual number of macrophages in a freshly prepared cell suspension.

Estimation of the Numbers of Macrophages in Mouse Spleen, Lymph Node or Thymus

Between 3×10^{x} and 1×10^{x} Balb/C spleen cells, 1×10^{x} and 3×10^{x} lymp node cells, or 3×10^{5} and 10×10^{x} thymus cells were plated in triplicate cultures. When counting LCC in day 3 cultures of cells from the three lymphoid organs no fibroblast-like cells were seen, whereas the day 7 cultures contained many of these cells. Table 5 shows the results of LCC counting and AO staining, respectively in one representative experiment. The calculated mean number of phagocytic cells among freshly prepared spleen cells (Table 5) was 3.49 per cent and among freshly prepared

TABLE 2 *The Mean per Cent of LCC[a] after 3 or 7 Days in Culture of PE Macrophages*

Days in culture	Numbers of PE macrophages plated per well[b]					
	1×10^4	2×10^4	6.7×10^3	4×10^3	2.2×10^3	8×10^2
3	5 0	8.9	9 7	6.1	9.8	3.9
7	3 8	6.3	12.2	2 8	9.3	1.3

a) See Table 1 b) and c)

b) Judged as seen in the light microscope (magnification = 400 ×)

7 experiments counted 7 days after culture The data obtained from the day 3 cultures were similar when between 2.2×10^3 and 2×10^4 PE macrophages were plated whereas the plating efficiency when more than 2×10^4 (1×10^4) or less than $2\ 2\times10^3$ (8×10^2) macrophages were plated was somewhat lower The same tendency was seen among the data from day 7 cultures although the results were more variable. Thus, it seemed that an exact reflection of the survival of macrophages in culture required plating more than 2×10^3 and less than 2×10^4 macrophage like cells per well in the culture system used here. Linear regression analysis of the paired data from the day 3 cultures, excluding the per cent LCC obtained by plating 8×10^2 or 1×10^4 PE macrophages, gave a straight line. Calculating the slope, s, gave $s = 0\ 1285$ The corresponding results from the day 7 cultures gave $s = 0\ 0859$ It was concluded that plating between 2×10 and 2×10^4 PE macrophages resulted in a survival of 10 7 per cent and thus, in a plating factor of 100/10 7 = 9.3

Effect of Anti-Θ Antiserum and Complement on the Survival of Phagocytes

Attempts at studying the effect of autologous macrophages on the generation of cytotoxic T lymphocytes (6) required knowledge of the *in vitro* behavior of anti-Θ antiserum plus complement treated macrophages. Theta positive depleted PEC were grown *in vitro* and their phagocytizing ability examined after different culture periods Table 3 shows the per cent of phagocytic cells related to the number of plated cells before or after treatment with anti-Θ antiserum and complement in one out of three experiments. This treatment did not affect the survival of phagocytic cells.

Acridine Orange Staining of PEC

Table 4 shows the per cent of macrophage-like cells in PEC from three different mouse strains as determined by acridine orange (the AO cells) The number of AO cells from CBA Balb/C × DBA or Balb/C mice was very similar and in all three cases more than

TABLE 3 *Per Cent LCC among Anti-Θ Antiserum and Complement Treated Balb/C PE Macrophages after 3 or 7 Days in Culture*

Days in culture	Anti-Θ + C[a]	Numbers of PE macrophages plated per well[b]			
		2×10^4	$6\ 7\times10^3$	2.2×10	$7\ 4\times10^2$
3	–	5 67	8.14	8.48	6.74
	+	6 63	6.53	5 15	4 04
7	–	11 67	8.43	13 09	7.33
		16 44	8 6?	7 08	2.63

a) – = cells treated with complement (per cent AO cells 78.6 per cent per cent EAC-rosette forming macrophages 21.5 per cent) + = *cells treated with anti-Θ antiserum plus c*[illegible] cells 83 0 per cent per cent EAC-rosette forming macrophages ?3 0 per cer

b) Judged as seen in the light microscope (*magnification = 400 ×*)

properties (development?) They also suggest that the composition of the mononuclear phagocytic group of cells in the lymph nodes is different from that of the spleen or thymus. Whether these differences appear in consequence of various developmental levels of the mononuclear phagocytic cells among lymph node cells or spleen and thymus cells was not determined.

It is pleasure to thank Dr *C. A. Janeway* for reading the manuscript and Dr *B. Rubin* for valuable advice. This work was supported by *Statens Lægevidenskabelige Forskningsråd* (J.no. 512 5875)

REFERENCES

1. *Bianco C. R., Patrick, R. & Nussenzweig, V.* A population of lymphocytes bearing a membrane receptor for antigen-antibody-complement complexes. J. exp. Med. *132* 702–720, 1970
2. *Cohn Z. A. & Benson B.* The in vitro differentiation of mononuclear phagocytes. II. The influence of serum on granule formation, hydrolase production, and pinocytosis. J. exp. Med. *121* 835–848, 1965.
3. *Fedorko M. E. & Hirsh, J. G.* Structure of monocytes and macrophages. Semin. Hematol. *7* 109–124 1970.
4. *Golstein P. & Blomgren, H.:* Further evidence for autonomy of T cells mediating specific in vitro cytotoxicity. Efficiency of very small amounts of highly purified T cells. Cell. Immunol. *9* 127–141 1973.
5. *Hertel-Wulff B. & Rubin, B.* Lymphocyte proliferation in vitro induced by soluble protein antigens. II. Cellular requirements. Eur. J. Immunol. *6* 418–424 1976.
6. *Hertel-Wulff B. & Rubin, B.:* T lymphocyte recognition of alloantigen in vitro. I. Significance of Fc receptor positive T and B stimulator cells in the generation of cytotoxic T lymphocytes. Scand. J. Immunol. (in press)
7. *Nelson, D. S.* (Ed.) Immunobiology of the macrophage. Academic Press. 1976.
8. *North R. J.* Endocytosis. Semin. Hematol. *7* 161–171 1970.
9. *Rubin B. & Hertel-Wulff B.* Biological significance of Fc receptor-bearing cells among activated T lymphocytes. Scand. J. Immunol. *4* 451–462, 1975
10. *Rubin, B. & Wigzell, H.* The immune response against hapten autologous protein conjugates in the mouse. III. Specificity of co-operating non-thymus processed (B) and thymus processed (T) lymphocytes. J. exp. Med. *137* 911 931 1973
11. *Vercleieren M. & Defendi V.* Dependence of macrophage growth in vitro upon interaction with other cell types. Wistar Institut Symposium Monograph on growth Regulating Substances for animal cells in culture. *7* 67–98, 1967

TABLE 5 *Per Cent Macrophage-like Cells in Spleen Lymph Node or Thymus Determined by Latex Particle Uptake after 3 Days in Culture or by Acridine Orange Staining*

Mouse strain	Spleen		Lymph node		Thymus	
	Per cent LCC	Per cent AO	Per cent LCC	Per cent AO	Per cent LCC	Per cent AO
Balb/C	3 49	4 24	0.055	1.39	0.13	0.25
CBA	4.98	4 68	0.098	2.07	0 44	0.4?

thymus cells (Table 5) 0 13 per cent, these two values being only slightly lower than the 4 24 per cent and 0.25 per cent of cells, respectively which stain with acridine orange (Table 5) Plating 3×10^{3} and 1×10^{6} lymph node cells gave a calculated mean of 0 055 per cent phagocytes among freshly prepared cells. The number of lymph node cells which stain with acridine orange (1.39 per cent) is more than twenty times higher The corresponding calculated numbers of Latex ingesting cells among spleen lymph node and thymus cells after 7 days in culture were 8.37 per cent 0 16 per cent and 0 15 per cent, respectively (not shown) The values from the spleen and lymph node cell cultures were more than doubled relative to day 3 Latex particle uptake probably owing to the great amount of fibroblast like cells found here. Plating cells from lymphoid organs of CBA mice gave similar results (Table 5)

DISCUSSION

The role of macrophages in immune reactions including their ability to cooperate with other immunologically important cell types has attracted much attention (7) The *in vitro* influence of freshly harvested macrophages on antigen recognition by T lymphocytes has been studied (7) as well as the effect of macrophages cultured for several days (6) Thus, the present study investigated whether it was possible to use two simple methods to obtain knowledge of the survival of phagocytic cells cultured from 3 to 7 days and, in addition of the number of macrophages in mouse spleen, lymph node and thymus. The methods used were the uptake of Latex particles by phagocytes, and the acridine orange technique in which lysosomes stain red. By culture of PEC from mice injected i.p. with IFA/BSS 5–7 days previously a survival of 12.8 per cent on day 3 and of 8.6 per cent on day 7 (mean 10 7 per cent) was obtained. This gave a plating factor of 9.3 This factor was used to calculate the number of phagocytes in three lymphoid mouse organs. The results obtained gave 3 49 per cent or 0.13 per cent phagocytes among spleen or thymus cells, respectively The per cent of phagocytes among lymph node cells was calculated to 0 06 per cent. Culturing cells from the lymphoid organs for 7 days resulted in the presence of fibroblast like cells, some of which were placed in colonies. Thus, results obtained from day 3 cultures were used for further studies. The calculated number of phagocytes among spleen (3 49 per cent) or thymus cells (0 13 per cent) correlated with the numbers of cells which stain with acridine orange (4.24 per cent or 0.25 per cent, respectively) while the number of phagocytes among lymph node cells was low related to the AO data (0 06 per cent LCC versus 1.39 per cent AO cells)

Thus, mouse spleen contains equal numbers of cells which phagocytize and which stain with acridine orange. This was valid for the thymus, too. By contrast, mouse lymph nodes contain cells which appear not to ingest Latex particles although they stain with AO It was concluded that the Latex and the AO techniques reflect the numbers of phagocytes in the mouse spleen or the mouse thymus. The results obtained using mouse lymph node cells indicate that this organ contains cells of the mononuclear phagocytic family with differing

properties (development?) They also suggest that the composition of the mononuclear phagocytic group of cells in the lymph nodes is different from that of the spleen or thymus. Whether these differences appear in consequence of various developmental levels of the mononuclear phagocytic cells among lymph node cells or spleen and thymus cells was not determined.

It is a pleasure to thank Dr C. A. Janeway for reading the manuscript and Dr B. Rubin for valuable advice. This work was supported by Statens Lægevidenskabelige Forskningsråd (J no. 512 5875)

REFERENCES

1. *Bianco C. R., Patrick R. & Nussenzweig, V.* A population of lymphocytes bearing membrane receptor for antigen-antibody-complement complexes. J exp. Med. *132* 702–720 1970

2. *Cohn, Z. A. & Benson B.:* The in vitro differentiation of mononuclear phagocytes. II. The influence of serum on granule formation, hydrolase production, and pinocytosis J exp. Med. *121* 835–848 1965

3. *Furth R. v. E & Hirsch J G.* Structure of monocytes and macrophages. Semin. Hematol. *7* 109–124 1970.

4. *Golstein P & Blomgren H.:* Further evidence for autonomy of T cells mediating specific in vitro cytotoxicity Efficiency of very small amounts of highly purified T cells. Cell. Immunol. *9* 127–141 1973.

5. *Hertel-Wulff B. & Rubin B.* Lymphocyte proliferation in vitro induced by soluble protein antigens. II Cellular requirements. Eur J Immunol. *6* 418–424 1976.

6. *Hertel-Wulff B. & Rubin, B.* T lymphocyte recognition of alloantigen in vitro. I Significance of Fc receptor positive T and B stimulator cells in the generation of cytotoxic T lymphocytes. Scand. J Immunol (in press)

7. *Nelson D S* (Ed.): Immunobiology of the macrophage Academic Press, 1976.

8. *North R. J.:* Endocytosis. Semin. Hematol. *7* 161–171 1970.

9. *Rubin B. & Hertel-Wulff B.* Biological significance of Fc receptor-bearing cells among activated T lymphocytes. Scand. J Immunol. *4* 451–462, 1975

10. *Rubin, B & Wigzell, H* The immune response against hapten autologous protein conjugates in the mouse. III. Specificity of cooperating non-thymus processed (B) and thymus processed (T) lymphocytes. J exp. Med. *137* 911–931 1973

11. *Virolainen, M & Defendi V.* Dependence of macrophage growth in vitro upon interaction with other cell types. Wistar Institut Symposium Monograph on growth Regulating Substances for animal cells in culture. *7* 67–98, 1967

Acta path. microbiol. scand. Sect. C, 85 260–266 1977

THE USE OF HOMOCARNOSINE AS A SPACER IN INDIRECT HAEMAGGLUTINATION

ARE B. DALEN

The Gade Institute, Department of Microbiology University of Bergen, Bergen, Norway

Dalen A. B The use of homocarnosine as a spacer in indirect haemagglutination. Acta path. microbiol. scand. Sect. C, 85 260–266 1977

Homocarnosine (γ-butyryl-L-histidine) was bound to erythrocytes through the action of glutaraldehyde in order to increase the number of available groups for diazotization on the surface of the cells. Diazotization was performed with tetrazotized di-o-anisidine (TOD) TOD decomposed rapidly at an alkaline pH. The reagent was stabilized by the use of borate buffers. The presence of homocarnosine on erythrocytes enhanced the coupling of histidine by diazotization. TOD formed bonds of varying lability with alifatic amino and imino groups. This inhibited completely the formation of stable bonds with histidine and tyrosine. The relative amounts of stable bonds formed by TOD and human serum albumin and TOD and γ-globulin varied inversely with the concentration of the proteins. The two proteins were used as model antigens and the presence of homocarnosine on the surface of the erythrocytes increased the sensitivity of the haemagglutination reaction by a factor of 4–8

Key words Homocarnosine spacer indirect haemagglutination.

A. B Dalen Mikrobiologisk avdeling Sentralsykehuset, N 7000 Trondheim, Norway

Received 3.ii 77 Accepted 2.iii.77

Much work has been done on the use of erythrocytes as carrier particles in the indirect haemagglutination reaction. Various antigens or haptens have been attached by coupling reagents, or simply by adsorption to tanned or to aldehyde fixed cells (4)

The obstacle of instability of the sensitized cells, which not only requires their immediate use but also creates problems of standardization, is avoided by the use of aldehyde fixed cells. Coupling of antigens to erythrocytes by means of diazo linkages has been carried out with bis-diazotized benzidine (5 10) Since it is cumbersome to prepare the reagents of defined quality the commercially available tetrazotized di-o-anisidine has been used for this purpose with success (2 3)

In this communication an attempt to increase the groups available for diazotization on the surface of erythrocytes is described. The method consisted of the attachment of homocarnosine (γ-aminobutyryl-L-histidine) to the cells by means of glutaraldehyde. Human serum albumin and human γ-globulin were used as model antigens and coupled to the erythrocytes by diazotization with tetrazotized di-o-anisidine (TOD) Conditions affecting this coupling were studied in some detail.

MATERIALS AND METHODS

Erythrocytes

Human O Rh erythrocytes were obtained from outdated bank blood which had been stored in sterile citrate dextrose solution for 4–5 weeks. Before use, the erythrocytes were washed four times in

6-10 volumes of phosphate buffered saline (PBS) pH 7.2. They were then packed at 1000 × g for 5 min.

Fixation of Erythrocytes

Glutaraldehyde (Serva, Heidelberg, West Germany) 25 per cent aqueous solution, was added to 4 per cent erythrocyte suspension in PBS to a concentration of 0.1 per cent. The suspension was stirred for 1 h at room temperature and washed three times in PBS. Quantities of homocarnosine varying from 1 to 50 mg per ml of packed erythrocytes were added in two ways, either after glutaraldehyde fixation for 1 h, centrifugation and resuspension of the erythrocytes (4 per cent) in PBS or during glutaraldehyde fixation after 30 min of incubation. In the latter case, the mixture of erythrocytes, glutaraldehyde and homocarnosine was stirred for another 30 min at room temperature.

Diazotization with tetrazotized di-o-anisidine (TOD)

TOD (Serva, Heidelberg, West Germany) was freshly prepared in distilled water at a concentration of 0.1 per cent (w/v). Various buffer systems were tested, borate buffer (0.1 M, pH 8.0–9.0) giving optimal conditions for coupling. For all trials 0.1 ml of packed erythrocytes were washed in the appropriate buffers. The erythrocytes were resuspended after centrifugation in 2.5 ml of the buffer. The antigens were added in small volume to the suspension and mixed. Finally TOD was added, and usually 0.4 ml of the 0.1 per cent aqueous solution was employed. The reaction was allowed to proceed at room temperature for 30 min with occasional shaking.

Agglutination Test

This was performed as described earlier (7) except that bovine serum albumin was added to the dextrose gelatin veronal (DGV) buffer to a final concentration of 0.5 per cent.

Spectrophotometric Analysis

A double-beam instrument, Unicam SP 800 Spectrophotometer was used (Unicam Instruments Ltd Cambridge). The reactions were performed at room temperature. Phosphate buffer, borate buffer and carbonate buffer were of the same molarity 0.1 M.

Chemicals

γ-globulin was 16.5 per cent preparation for human use (AB Kabi, Stockholm, Sweden) and human serum albumin (HSA) and rabbit anti-human IgG was obtained from Behringwerke (Marburg-Lahn, West Germany). The amino acids and the imidazole histidine derivatives were obtained from Sigma Chemical Co. (St. Louis, Mo., USA).

RESULTS

Binding of Homocarnosine to Erythrocytes

The binding of homocarnosine was efficient when the dipeptides were added during fixation with glutaraldehyde. The highest concentrations of homocarnosine tested (50 mg per 1 ml packed cells) produced erythrocytes which showed a pronounced tendency to spontaneous agglutination. The maximal amount of homocarnosine suitable for binding to erythrocytes was found to be 20 mg per ml of packed cells. At this concentration no homocarnosine could be detected in the supernatant after binding in the presence of free glutaraldehyde.

Binding of homocarnosine to erythrocytes after fixation with glutaraldehyde and washing of the erythrocytes was considerably less efficient. About 50 per cent of the homocarnosine remained unbound if 20 mg homocarnosine per ml packed cells was employed.

The Reaction of TOD with Amino Acids

The reaction of TOD with the 19 commonly occurring L-amino acids was tested by dissolving the amino acids in borate buffer pH 9.0 (0.25 mg/ml) and adding TOD to a final concentration of 0.01 per cent. A yellow colour appeared (λ maximum, 425–450 nm) after a varying length of time. The stained reaction products were unstable, and the colour faded at a varying rate. Tyrosine, histidine and tryptophan were an exception to this. These amino acids yielded stable reaction products with a λ maximum of tryptophan 375 nm, histidine 436 nm and tyrosine 450 nm. The unstable, coloured compound was probably formed by a reaction of TOD with the amino groups, and this was verified by observing the colour formation of derivatives of butyric acid (Fig. 1). No colour was produced with isobutyric acid. The location of the amino groups in the α or γ-positions did not influence colour formation, nor did the presence of a hydroxyl group in a vicinal position affect it.

The rate of reaction of TOD with the amino groups varied considerably as illustrated in

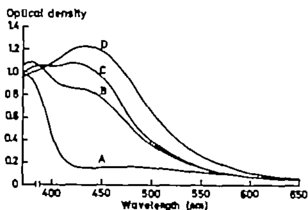


Fig 1 Absorption spectra of the reaction products of TOD and various derivatives of butyric acid, examined in borate buffer pH 9.0 0 1 M. A Isobutyric acid, B L-β-hydroxy-α-amino-butyric acid C L-α aminobutyric acid D L-γ-amino-butyric acid

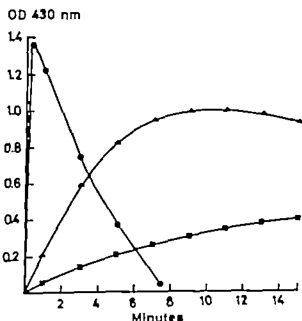


Fig 2 Reaction of TOD with proline (—●—) L-aspartic acid (—▲—) and L-leucine (—■—) in borate buffer (pH 9 0 0 1 M)

Fig 2 The most rapid reaction took place with the imino groups of proline and hydroxyproline, which developed within seconds, and disappeared at a corresponding rate. The labile yellow colour observed with the sixteen amino acids disappeared within a few hours, and if fresh TOD was added the yellow colour reappeared indicating an unmodified amino group after the reaction.

TABLE 1 *Absorbance Maxima of the Stable Compounds Formed by TOD and Various Histidine and Tyrosine Derivatives (pH 9.0)*

	λ maximum (nm)
L-histidine	436
N acetyl histidine	474
L-histidyl-glycine	458
L-glycyl histidine	472
Homocarnosine	480
Imidazole acetic acid	480
Histamine	480
L-tyrosine	450
L-glycyl-tyrosine	430

The reactivity of TOD towards various histidine and tyrosine derivatives was tested (Table 1) A stable colour was formed indicating stable bonds as in the case of the free amino acids. The λ maximum shifted towards a longer wavelength when the α-amino or carboxyl group of histidine was involved in the peptide linkage A shift towards a shorter wavelength appeared with the tyrosine dipeptide.

The Effect of the Buffer and pH on the Reactivity of TOD

TOD decomposed spontaneously in aqueous solution with the formation of a red-brown coloured compound. The rate of decomposition increased with increasing pH (Fig 3) However a slower reaction was noted in borate buffer indicating an influence of the anion on the rate of reaction. This was further explored by comparing the spontaneous decomposition of TOD and its reactivity with homocarnosine at pH 8.0 in a borate and a carbonate buffer (Fig 4) The spontaneous decomposition of TOD was markedly slower in the borate system. The rate of colour formation due to the reaction with homocarnosine was slower and attained a higher level in the borate system indicating a more complete reaction. Maximal colour formation in borate buffer appeared after 35 min at pH 8.0 and 13 min at pH 9 0

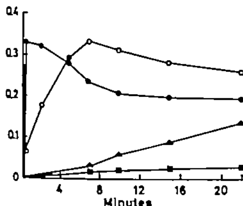


Fig. 3. Spontaneous decomposition of TOD (0.01 per cent) in various buffers (0.1 M). —▲— phosphate buffer pH 7.0. —■— borate buffer pH 8.0, —○— carbonate buffer pH 9.0. —●— carbonate buffer, pH 10.0.

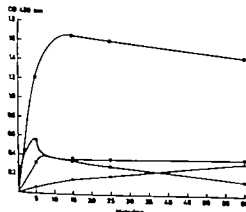


Fig. 4. Reactions of TOD (0.1 ml, 0.1 per cent) at pH 9.0. —▲—borate buffer. —■— carbonate buffer. —○— borate buffer with homocarnosine (0.25 mg/ml). —●— carbonate buffer with homocarnosine (0.25 mg/ml). Absorbance was measured at 480 nm.

Inhibition of Stable Diazo-bond Formation of Histidine by Imino-groups

The reactivities of TOD with homocarnosine and tryptophan, and with homocarnosine and proline in mixtures were compared. The concentration of TOD was varied between 0.05 per cent and 0.25 per cent, and absorbance at 382 nm and 482 nm was measured. When in mixture, an equal reactivity of TOD towards tryptophan and homocarnosine was demonstrated. In contrast, it was found that proline inhibited the colour formation of TOD with the histidine of homocarnosine, indicating an inhibition of the stable diazo-bond formation. The inhibition was partially overcome by increasing the concentration of TOD but an inhibition of 85 per cent was still observed at a concentration of TOD of 0.025 per cent.

The Relations of TOD with HSA and γ-globulin

An efficient sensitization of erythrocytes requires a stable diazo-bond formation with both the antigen and the erythrocytes. This was controlled by observing the colour formation with γ-globulin and HSA, alone and in mixtures with homocarnosine. The absorbance at 470 nm was used as an indicator of

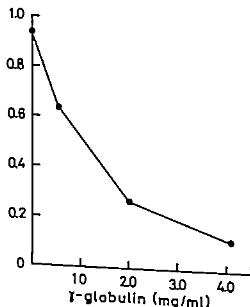


Fig. 5. The inhibitory effect of γ-globulin on the diazotization of the histidine group of homocarnosine (0.25 mg/ml). The degree of diazotization is indicated by the absorbance at 484 nm.

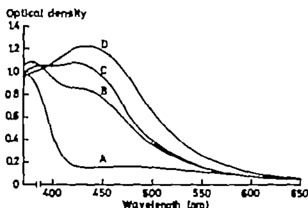


Fig 1 Absorption spectra of the reaction products of TOD and various derivatives of butyric acid, examined in borate buffer pH 9.0 0.1 M. A isobutyric acid, B L-β-hydroxy-α-amino-butyric acid, C L-α-aminobutyric acid D L γ-amino-butyric acid.

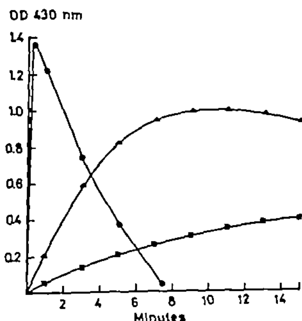


Fig 2 Reaction of TOD with proline (—●—) L-aspartic acid (—▲—) and L-leucine (—■—) in borate buffer (pH 9 0 0 1 M)

Fig 2. The most rapid reaction took place with the imino groups of proline and hydroxyproline, which developed within seconds and disappeared at a corresponding rate. The labile yellow colour observed with the sixteen amino acids disappeared within a few hours, and if fresh TOD was added the yellow colour reappeared indicating an unmodified amino group after the reaction.

TABLE 1 *Absorbance Maxima of the Stable Compounds Formed by TOD and Various Histidine and Tyrosine Derivatives (pH 9.0)*

	λ maximum (nm)
L-histidine	436
N-acetyl histidine	474
L histidyl-glycine	438
L-glycyl histidine	472
Homocarnosine	480
Imidazole acetic acid	480
Histamine	480
L-tyrosine	430
L-glycyl tyrosine	430

The reactivity of TOD towards various histidine and tyrosine derivatives was tested (Table 1) A stable colour was formed indicating stable bonds as in the case of the free amino acids. The λ maximum shifted towards a longer wavelength when the α-amino or carboxyl group of histidine was involved in the peptide linkage. A shift towards a shorter wavelength appeared with the tyrosine dipeptide.

The Effect of the Buffer and pH on the Reactivity of TOD

TOD decomposed spontaneously in aqueous solution with the formation of a red-brown coloured compound. The rate of decomposition increased with increasing pH (Fig 3) However a slower reaction was noted in borate buffer indicating an influence of the anion on the rate of reaction. This was further explored by comparing the spontaneous decomposition of TOD and its reactivity with homocarnosine at pH 8.0 in a borate and a carbonate buffer (Fig 4) The spontaneous decomposition of TOD was markedly slower in the borate system The rate of colour formation due to the reaction with homocarnosine was slower and attained a higher level in the borate system indicating a more complete reaction. Maximal colour formation in borate buffer appeared after 35 min at pH 8.0 and 13 min at pH 9 0

erythrocytes. The erythrocytes fixed in glutaraldehyde fulfil the requirement of stability (7). Due to its bifunctional properties, glutaraldehyde has been used both as a fixative and a coupling reagent (2, 9). A suitable coating is obtained with some protein antigens while others, i.e. γ-globulin, are less efficiently attached. An additional system for coupling of protein antigens to glutaraldehyde-fixed erythrocytes would therefore be required in many instances. For this purpose, diazo reagents have been found suitable (3). There are available groups for diazotation, both on native and on glutaraldehyde-fixed cells (3). A double fixation of erythrocytes with glutaraldehyde and sulphosalicylic acid has been shown to improve the binding of cell protein antigen to the surface by diazotization (3). From experience with affinity chromatography it has become clear that the ligand groups, critical in the interaction with the macromolecule to be purified, must be sufficiently distant from the backbone of the solid matrix to minimize steric interference with the binding process (6). This has been accomplished by introducing various "spacers" between the ligand and the solid support. There is little available information on the effect of steric hindrance in indirect haemagglutination, but it is thought to be of importance. Homocarnosine was chosen as a spacer with the dual purpose in mind of increasing the number of available groups for diazotation on the surface of the erythrocytes and of minimizing steric hindrance to the antigen/antibody reaction. The ε-amino group of lysine is particularly involved in the reaction of glutaraldehyde with protein (1). By analogy the γ-amino group of homocarnosine would be expected to react in the same manner. The binding of homocarnosine by glutaraldehyde to erythrocytes would then orient the imidazole group of homocarnosine away from the erythrocyte surface. A binding of homocarnosine to erythrocytes was observed. The coupling of histidine to erythrocytes by diazotisation was enhanced in the presence of bound homocarnosine. Attempts to demonstrate the same with protein antigens were unsuccessful due to interference of the diazo reagent with the staining reactions for proteins. A marking of the proteins with a radio-isotope would be required to establish this relation. The stabilizing effect of the borate buffer on TOD was convincing, and the degree of diazotization of histidine both in its free form and as a part of peptides was higher in borate buffer than in carbonate buffer under otherwise identical conditions. The rate of reaction could be controlled by adjusting the pH.

The formation of labile bonds and inactivation of TOD by imino and alifatic amino groups indicated the limitations of the coupling procedure. For instance, the presence of free amino acids and notably proline and hydroxyproline in a protein antigen preparation would interfere with the diazocoupling of the antigen to erythrocytes. The ratio in a particular protein of the rapidly reacting amino acid residues to the more slowly reacting histidine and tyrosine residues would determine the number of stable bonds formed between the protein and the erythrocyte surface. This was clearly demonstrated by HSA and γ-globulin, HSA showing a more extensive reaction with histidine residues than γ-globulin. These findings are in agreement with the observation of *Haber and Rose* (8) of a constant bis-diazobenzidine-antigen ratio for optimal attachment of proteins to erythrocytes. The optimal concentration of TOD should be determined for the protein antigen in question. This could be assessed by measuring the absorbance at the appropriate wavelength, indicating a reaction with histidine or tyrosine residues. A comparison of this with the absorbance of reaction products of TOD and mixtures of homocarnosine and the protein antigens seemed to indicate that there had occurred stable binding of the antigens to the homocarnosine-carrying erythrocytes. Due to the conspicuous colour formed by diazotization of histidine, this could be done by simple visual observation.

The protein antigens tested were both bound to a certain extent to erythrocytes by TOD through the α-amino groups of amino acids. These bonds would be expected to be

a stable diazo-bond with histidine. Comparing equal concentrations of HSA and IgG showed a higher absorbance of HSA at this wave length indicating a more extensive reaction with intramolecular histidine in HSA If the concentration both of HSA and γ-globulin was reduced a relative increase in the absorbance at 470 nm took place indicating an increased reactivity with the histidine groups of the molecule. HSA and especially γ-globulin showed a clear inhibitory effect of the diazotization of homocarnosine (Fig 5) It was partially overcome by increasing the concentration of TOD

The Binding of Histidine to Homocarnosine Treated Erythrocytes

The binding of histidine by diazotization to glutaraldehyde-treated and homocarnosine-containing cells was compared by measuring unreacted histidine in the supernatant by the absorbance at 436 nm after the reaction. At all concentrations of histidine the homocarnosine-treated cells bound more of the amino acid HSA and γ-globulin were removed from the supernatant during the diazotization process even at high concentrations of the proteins This could be due to labile bindings through α-amino groups on the proteins. The elution of HSA and IgG from the erythrocytes on storage was not tested

The Coupling of HSA and IgG to Erythrocytes Treated with Homocarnosine

The presence of homocarnosine on the erythrocytes increased the titres in both test systems by a factor of 4 to 8 compared to that of glutaraldehyde fixed cells. The optimum amount of homocarnosine was found to be 20 mg per ml packed cells. The patterns developed within 3–4 h at room temperature, and they were easy to read.

The Effect of pH and Buffer on the Coupling to Homocarnosine-Treated Cells

The effect of pH and buffer type on coupling of the HSA to erythrocytes was analogous to the effect seen with diazotization of amino acids (Table 2) Similar results were obtained

TABLE 2 *The Relation of the Anti-HSA Titre to the Buffer Employed in the Coupling of the Antigen to Erythrocytes Carrying Homocarnosine*

Buffer	Anti-HSA serum titres (−log²)
phosphate	
pH 7.0	10
pH 7.5	9
borate	
pH 8.0	15
pH 8.5	15
carbonate	
pH 9.0	11
pH 9.5	11

using γ-globulin. The use of borate buffer gave significantly higher titres. This was less pronounced with IgG than with HSA.

The Effect of Antigen Concentration on the Coupling Reaction

As seen in Table 3 high concentrations of antigen reduced the sensitivity of the reaction. This would be the expected consequence of the inhibition by high concentrations of protein on the formation of stable diazo-bonds noted earlier

TABLE 3 *Anti-HSA Titres Obtained with Different Amounts of HSA Conjugations Were Performed in Borate Buffer pH 9.0 with 0.1 ml Packed Erythrocytes Containing 20 mg Homocarnosine/ml Packed Cells*

HSA (mg)	Anti HSA serum titres (−log²)
2	12
5	14
10	15
20	16
50	15
100	13

DISCUSSION

The ideal requirements for indirect haemagglutination would be erythrocytes of infinite stability and with minimally modified antigens covalently attached to the surface of the

Acta path. microbiol. scand. Sect. C, 85 267-276 1977

ANTIBODY DEPENDENT CELL-MEDIATED CYTOTOXICITY BY ACTIVATED THYMOCYTE POPULATIONS

Nature of the effector cell(s)

BENT RUBIN and M. HOIER MADSEN

Immunobiology Laboratory Statens Seruminstitut, Copenhagen, Denmark

Rubin, B. & Høier Madsen, M. Antibody-dependent cell-mediated cytotoxicity by activated thymocyte populations. Nature of the effector cell(s) Acta path. microbiol. scand. Sect. C, 85 267 276, 1977

The results of the present experiments have shown that activated thymocyte populations are endowed with cytotoxic potential against antibody-coated target cells. The effector cells could be divided into at least two subpopulations 1) host-derived macrophage-like cells and 2) thymocyte-derived donor cell T lymphocytes. These two subpopulations could be separated and identified by means of commonly used immunofractionation techniques. Such data show that effector cells which kill antibody-coated target cells may be of T cell nature.

Key words Cell-mediated cytotoxicity antibody-dependent activated thymocyte population.

B Rubin, Immunobiology Laboratory Statens Seruminstitut,
Amager Boulevard 80 DK 2300 Copenhagen S, Denmark.

Received 3 77 Accepted 6.iii.77

Normal thymus cells injected into lethally irradiated syngeneic mice (plus an antigen) or into lethally irradiated semiallogeneic (F1) mice and harvested from the spleen of the irradiated hosts are defined as activated thymocytes (ATCs) When thymocytes are activated in a semiallogeneic environment, parental thymocytes develop into cytotoxic T cells specific for the H 2 antigens possessed by the other parent (1 6, 28) When thymocytes are activated in a syngeneic environment and immunized with a protein antigen specific helper T cells develop (22 23) Regardless of the activation procedure ATCs contain a relatively high proportion of Fc receptor (F R) bearing cells (20-90 per cent) However neither cytotoxic T cells (CTL) helper T cells nor phytohaemagglutinin (PHA) responding T cells among ATCs seemed to express FcR in their effector state (6, 22 23) We asked the question whether θ positive, FcR T cells among ATCs were effector cells in the antibody-dependent cell-mediated cytotoxic reaction (ADCC) This question was based on previous findings which suggested the existence of some ADCC activity in ATC populations (4, 23)

Among the cell types known to be active in this reaction are 1) lymphocytes (7 8, 17) 2) macrophages (5 8, 32) 3) granulocytes (5) and non-lymphoid tumour cells (15) Since ATC populations consist of more than 80 per cent θ positive cells, less than 5 per cent immunoglobulin (Ig) positive or C3 receptor (C3R) positive cells, 20-90 per cent

labile. The elution on storage of antigens bound in such a manner was not studied. The formation of such labile bonds would explain the findings that the use of two different coupling principles in succession yielded erythrocytes showing a higher degree of sensitization (3). Preliminary studies using various antigens bound to erythrocytes by diazotization followed by application of carbodiimides have supported this observation.

REFERENCES

1. *Avrameas S. & Ternynck T.*, The crosslinking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents. Immunochemistry *6* 53–66 1969
2. *Avrameas S. Taudou B. & Chuilon S.* Glutaraldehyde, cyanuric chloride and tetrazotized o-dianisidine as coupling reagents in the passive hemagglutination test. Immunochemistry *6* 67–76 1969
3. *Becht H. & Malole B.* Comparative evaluation of different fixation procedures and different coupling reagents for the demonstration of influenza virus-specific antibodies by the indirect hemagglutination test. Med. Microbiol. Immunol. *162* 43–53 1975
4. *Boguslaski R. C. Smith R. S. & Mhatre N. S.* Applications of bound biopolymers in enzymology and immunology. In: Current Topics in Microbiology and Immunology vol. 58 Springer Verlag Berlin–Heidelberg–New York 1972, pp. 1–68
5. *Butler W. T.* Hemagglutination studies with formalinized erythrocytes. Effect of bis-diazobenzidine and tannic acid treatment on sensitization by soluble antigens. J. Immunol. *90* 663–671 1963
6. *Cuatrecasas P.*, Protein purification by affinity chromatography. Derivations of agarose and polyacrylamide beads. J. biol. Chem. *245* 3059–3065 1970
7. *Dalen A. B.* The effect of glutaraldehyde on the stability of erythrocytes and on virus receptor substances. Acta path. microbiol. scand. Sect. B *84* 196–200 1976
8. *Haber J. A. & Rose N. R.* Comparative studies of indirect hemagglutination. Int. Arch. Allergy *34* 303–311 1968.
9. *Onkelinx E. Meuldermans W., Joniau M. & Lontie R.* Glutaraldehyde as a coupling reagent in passive haemagglutination. Immunology *16* 35–43 1969
10. *Stavitsky A. B. & Arquilla, E. R.*, Micromethods for the study of proteins and antibodies. III. Procedure and applications of hemagglutination and hemagglutination-inhibition reactions with bis-diazotized benzidine and protein-conjugated red blood cells. J. Immunol. *74* 306–312 1955

interface cells were harvested. They contained less than 5 per cent dead cells. When tested in the ADCC assay it was found that this procedure removed the inhibitory antigen-antibody complexes (and other inhibitory substances) from the control group of cells treated with complement or antibody alone (Table 1)

Characterization of cells As already mentioned, T cells were identified by the anti-θ antiserum and complement cytotoxicity test, B cells were identified by rosette assays using EA (E = sheep erythrocytes (SRBC) A = 7S mouse anti-SRBC) or EAC' (E = SRBC, A = 19S rabbit anti-SRBC, C' = C5 deficient mouse serum) complexes (11 22) or by indirect immunofluorescence (6, 22) Macrophages were determined by the acridine orange technique (7)

Experimental design Mice were killed at different intervals after irradiation and thymocyte injection and their spleen cells harvested. They were then tested for ADCC activity T cell-mediated cytotoxicity and content of T B, and macrophage-like cells.

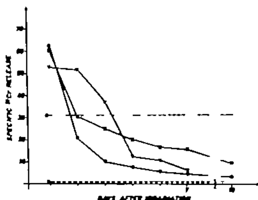


Fig 1 Cytotoxicity against Ab-CRBC by different kinds of "activated" thymocytes.
Spleen cells from group I (800 R Balb/cxCBA) group II (800 R Balb/cxCBA mice injected with 10^8 Balb/cxCBA thymocytes) and group III (800 R Balb/cxCBA mice injected with 10^8 CBA thymocytes) were harvested from day 2 to day 10 following irradiation and injection. The results are the means of four independent experiments. Per cent specific ^{51}Cr release at a target effector cell ratio of 1.5 is plotted against days after irradiation. Group I = o——o group II = □——□ group III = ▽——▽ The cytotoxic potential of spleen cells (●) (the housing organ) in ratio 1.5 and of thymocytes (▼) (the donor cells) in ratio 1.50 is also shown (——)

RESULTS

Development of ADCC Activity among ATCs

From day 2 to day 10 five mice per group were killed and spleen cell suspensions were tested in the ADCC assay with normal spleen and thymus cells as controls. The pooled results of four experiments are given in Fig. 1 On day 2 the ADCC activity was relatively high in all three groups, after which it decreased. The cytotoxic activity shown by group I ATCs represents the background noise of the system. Thus, both group II and III ATCs displayed a cytotoxic activity significantly above background on days 3 and 4 after irradiation. There was significantly higher cytotoxic activity of group II ATCs compared with background on days 5 6, and 7 whereas group III ATCs were not more cytotoxic than background on days 5–7

Mediators of ADCC Activity Host and/or Donor Type Cells?

Since the experiments in the preceeding section showed that spleen cells from irradiated mice displayed ADCC activity it was important to establish whether the ADCC activity among ATCs (group II and III) was donor and/or host-derived. Two types of experiments were performed 1) Balb/cxCBA thymocytes were activated in 800 R CBA mice The ADCC activity should be sensitive to CBA anti-Balb/c antiserum and complement only if it was of donor origin. The results in Tables 1 and 2 show that treatment with this antiserum and complement reduced the ADCC activity at least 10-fold. 2) C57 thymocytes were activated in 800 R Balb/cxC57 mice. If host cells contributed significantly to the ADCC activity of ATCs, this activity should be sensitive to C57 anti-Balb/c antiserum and complement. The results in Table 3 show that the ADCC activity was reduced about 5-fold, whereas the T cell cytotoxic activity of the same cell population was slightly enhanced. Thus both donor and host derived cells contribute to the ADCC activity of ATCs.

FcR cells 2–10 per cent macrophages (and monocytes) and probably also some granulocytes (4 22) it was important to classify the ADCC effector cell(s) among the ATCs. By using different fractionation procedures as well as lymphocytotoxicity assays with anti H2 antibody and complement, we obtained results which suggest that ADCC in ATC populations was mediated by two different types of effector cells 1) Host-derived macrophage-like cells and 2) donor type thymocyte-derived T cells

MATERIALS AND METHODS

Animals C57Bl/6 Balb/c, CBA/J and A.SW in bred mice and their F1 hybrids were used in the present study Outbred rabbits were used to raise antisera for the immunoadsorbent columns (22 24) Chickens provided red blood cells (CRBC)

Activation of thymocytes Recipient mice were irradiated with 800 R as described previously (22) Usually 30–60 irradiated F1 hybrid mice were divided into three groups group I irradiation controls group II mice injected with 10^8 syngeneic thymocytes group III mice injected with 10^8 semiallogeneic thymocytes. In experiments designed to establish the origin of ADCC effector cells use was made of irradiated parental (a) as well as F1 hybrid (axb) mice These mice were injected with 10^8 F1 (axb) and 10^8 parental (a) thymocytes, respectively Treatment of cell suspensions from such mice with an a anti b alloantiserum and complement will kill donor-derived cells in the former case and host-derived cells in the latter case. Although certain of the above-mentioned activation procedures will produce specific cytotoxic effector cells (group III (6 23 28) and F1 anti parental in which Hh-1 differences exist (27)) whereas others will not, all the different cell populations obtained from irradiated mice will be considered as ATCs. The reason is that all such cell populations have similar proportions of FcR cells, even cells from irradiation controls (22)

Antibody-dependent cell mediated cytotoxicity assay (ADCC assay) This assay has been described in detail elsewhere (24) In short, ATC spleen cells or normal spleen and thymus cells were harvested in 10 per cent foetal calf serum/F13 Eagles minimum essential medium (FCS/F13) and single cell suspensions prepared as described (22) Erythrocytes were lysed with 0 84 per cent NH Cl. Other fractionation procedures were then applied as described below The last step before the ADCC assay was centrifugation through FCS (31) To each ADCC assay tube the following were added 5×10^4 ^{51}Cr labelled CRBC, rabbit 7S anti-CRBC antibody diluted 1 10^4 and effector cells in ratios of 1–50 1 CRBC The total incubation volume was 1 ml of FCS/F13 The mixtures were incubated for 18 hours at 37 C/5 per cent CO_2 and then tested for radioactivity using liquid scintillation counting in a Nuclear Chicago Isocap apparatus. Percentage ^{51}Cr released was calculated by the formula $(2a/a+b) \times 100$ where a = cpm in half the supernatant and b = cpm in half the supernatant plus pellet. Percentage specific ^{51}Cr release was determined by subtracting per cent ^{51}Cr released by effector cells in the absence of anti-CRBC antibody from per cent ^{51}Cr released by effector cells in the presence of anti-CRBC antibody Spontaneous release of ^{51}Cr from CRBC was less than 5 per cent. ^{51}Cr release in the presence of effector cells without antibody was never more than 8 per cent. Only specific ^{51}Cr release is shown in the tables and figures.

Differences in ADCC activity between different ATC populations or between non-fractionated and fractionated ATC populations are expressed as the number of cells of one population needed to cause e.g 25 per cent specific ^{51}Cr release from Ab CRBC in comparison with the number of cells of another population needed to cause 25 per cent ^{51}Cr release from Ab-CRBC. Thus, the term "th ADCC activity was reduced 10-fold by procedur " means that after the procedure it is necessar to use 10 times more effector cells from the fra tionated ATC population compared with the non fractionated ATC population in order to get 25 pe cent specific ^{51}Cr release (i.e. a relative measurement rather than an absolute measurement)

Direct T cell mediated cytotoxicity assay T cel mediated cytotoxicity was determined against a in vitro maintained line of P815 (a DBA/2 mast cytoma) as described previously (6 24)

Fractionation procedures The following proc dures have been described in detail elsewher Fractionation on columns coated with Ig anti complexes (24) adsorption on a plastic surfa (9) treatment with carbonyl-iron and magnet (9) T cells were removed by treatment with anti antiserum and complement (see below)

Anti H2 antisera were produced and cell suspe sions were treated with these antisera or anti-θ a complement as described previously (7 11 15 However since cells treated with antibody alo would create antigen-antibody complexes wh might inhibit the ADCC assay (7) the follow procedure was adopted Cell suspensions were treated with medium, complement, antibody or antibody and complement and then washed once through FCS. Then aliquots of the four groups of cells were cultured for three hours at 37 C/5 per cent CO_2 $(10 \times 10^6/ml)$ washed once and then applied onto a Ficoll Isopaque (FIP) gradient, as described by *Parish et al.* (16) They were centr fuged for 30 minutes at $2\,000 \times g$ (22 C) and

TABLE 3. *Effect of Alloantiserum Directed against the Host C lls on the ADCC and T Cell-mediat d Cytotoxic Activity of ATCs*

Effector cells)	Antiserum b)	Per cent specific ⁵¹Cr release from)					
		Ab-CRBC				P815	
		1:0.2	1:1	1:5	1 25	1:2	1 10
(Balb/cxC57) anti-Balb/cxC57 ATCs (I)	—	2.8	6.9	16.2	32.7	0.9	—3 1
— —	Anti-Balb/c	12.5	23.3	—	—	—	—
C57 anti-Balb/cxC57 ATCs (II)	—	—	7.9	18.5	29.5	34.8	69.2
— —	Anti-Balb/c	—	7.0	13.2	18.7	37 4	71.5

a) Effector cells were harvested on day 5 of *in vivo* activation.
b) — = cells treated with complement alone. Anti-Balb/c = cells treated with anti-Balb/c antiserum and complement (see also Table 1) Cell recovery after treatment I = 5 8 per cent, II = 93.9 per cent.
c) 1 0.2, 1 1 = target:effector cell ratio. Spontaneous release Ab-CRBC = 5.1 per cent, P815 = 8.1 per cent. — = not done

TABLE 4. *Effect of Anti-θ Antiserum and Complement on the Cytotoxic Potential of ATCs against Ab-CRBC and P815 Tumour Cells*

Effector cells)	Antiserum b)	Per cent specific ⁵¹Cr release from)					
		Ab-CRBC			P815		
		1 1	1:5	1.25	1·0 4	1:2	1 10
(Balb/cxCBA) anti-Balb/cxCBA ATCs (I)	C'	9.2	17 1	34 8	0.2	—3.2	—4 1
—	Anti-θ	3 7	8.2	18 8	0.7	—3 8	—4.2
CBA anti-Balb/cxCBA ATCs (II)	C'	5.6	16 5	27.8	23.8	48.2	60.7
— —	Anti-θ	2.1	4.9	10.8	—2.0	0 8	8.8

) Effector cells harvested on day 4 of in vo activation. Per cent θ positiv cells I = 77.5 II = 91.9 Per cent EA RFC I = 43.8, II = 39 7
b C' = cells treated with complement. Anti-θ = cells treated with anti-θ antiserum and complement. For further details, see Table 1
c) 1 1 1.5, = target:effector cell ratio. Spontaneous release Ab-CRBC = 2.8 per cent, P815 = 5.7 per cent

that removal of such cells from ATCs resulted in a 5–10 fold reduction of ADCC. On the other hand, T cell cytotoxicity against P815 was somewhat enhanced. Thus, ADCC is either also mediated by adherent/phagocytic cells or mediated by other cells which are dependent on macrophage-like cells (see Discussion)

Is ATC-ADCC Activity Performed by Two Types of Effector Cells?

It would seem logical from the preceeding results that the θ positive ADCC effector cells were donor-derived and the adherent ones were host-derived. This hypothesis was tested by combining the adherence fractionation technique with the treatment with alloanti-

TABLE 1 *Cytotoxicity against Ab-CRBC of (Balb/cxCBA) Anti-CBA ATCs*)

Target Effector cell ratio	Without 3 hour pre-culture b) F13	C'	Anti-Balb/c	Anti-Balb/c + C'	With 3 hour pre-culture c) F13	C'	Anti-Balb/c	Anti-Balb/c + C'
1 25	35.9	28 1	10.3	12.3	36 2	32.1	33.0	13.7
1 5	21 8	13 1	8.1	7.9	23.9	21.0	19.2	8.3
1 1	9 7	8.3	3.2	4.3	12.4	9 8	8.3	3.6

a) Results are expressed as per cent specific ^{51}Cr release. Effector cells = spleen cells from 800 R CBA mice injected with 10^8 Balb/cxCBA thymocytes (harvested on day 5)

b) After treatment the cells were washed twice and centrifuged on FIP (see Materials and methods)

c) After treatment the cells were washed twice and incubated for 3 hours at 37 C/5 per cent CO_2. The cells were then washed once and centrifuged on FIP

TABLE 2 *Effect of Alloantiserum Directed against the Injected Thymocytes on the ADCC Activity of Different Types of ATCs*)

Target Effector cell ratio	Effector cells b) N-CBA SpL		CBA anti-CBA ATCs		(Balb/cxCBA) anti-CBA ATCs	
antiserum)	—	Anti-Balb/c	—	Anti Balb/c	—	Anti-Balb/c
1 25	46 4	39 7	28.6	23.2	29.0	11.3
1 5	12.9	17 8	2[illegible].5	21 8	17.5	7 1
1 1	3.5	5 1	18.3	17.5	8.9	3.9
Cell recovery		85.2 %		91.5 %		38.1 %

a) Results are expressed as per cent specific ^{51}Cr release.

b) N CBA SpL = normal CBA spleen cells. CBA anti-CBA ATCs = spleen cells from 800 R irradiated CBA mice injected with 10^8 CBA thymocytes. (Balb/cxCBA) anti-CBA ATCs = spleen cells from 800 R irradiated CBA mice injected with 10^8 Balb/cxCBA thymocytes. Cells were harvested on day 5.

c) — = cells treated with FCS/F13 and complement. Anti Balb/c = cells treated with anti-Balb/c antiserum and complement. The treated cells were incubated before ADCC assay as described in Table 1.

d) Calculated in relation to the complement control. Complement alone gave about 85 per cent recovery in all three groups.

Is ADCC Performed by T Cells?

By using an experimental design similar to that described in the previous section, we investigated whether or not the ADCC activity was mediated by T cells. The results in Table 4 show that anti-Θ antiserum and complement reduced the ADCC activity of group II and group III ATCs 5–10 fold. At the same time, the T cell cytotoxicity of group III cells was almost completely reduced. Thus, a considerable part of the ADCC activity was performed by Θ positive cells.

Is ADCC Performed by Adherent and/or Phagocytic Cells?

Since most of the cells surviving 800 R irradiation would be cells of the monocyte/macrophage series, we determined whether ADCC was mediated by adherent and/or phagocytic cells. The results in Table 5 show

TABLE 7 *Effect of Ig Anti-Ig Column Passag on the Cytot xic Potential of ATCs against Ab-CRBC*

Effector cells)	Ig anti-Ig column passage in b)	% EA-RFC	Per cent specific 51Cr release from) Ab-CRBC 1 1	1.5	1.25
(Balb/cxCBA) anti-Balb/cxCBA ATCs (I)	—	43 4	3.7	9.5	23.0
–"–	+	0 5	—0 9	0.5	1.9
– –	— (EDTA)	42.8	4 1	8.7	24.8
–	+ (EDTA)	0.2	0.5	3.3	7.1
CBA anti-Balb/cxCBA ATCs (II)	—	51.5	8.5	16.5	38.9
– –	+	0.4	0.2	0.6	4.4
– –	— (EDTA)	55 6	7.9	14.9	36.1
– –	+ (EDTA)	0 8	0 1	0.5	6.1

a) Effector cells were harvested on day 4 Per cent Θ positiv cells I = 90.5 II = 97.2.
b) ATCs were non-passed (—) or passed (+) through Ig anti-Ig columns in absence or presence (EDTA) of EDTA-FCS/F15
c) 1 1 1.5 target-effector cell ratio. Spontaneous release from CRBC = 2.7 per cent, from CRBC (EDTA) = 2.6 per cent.

TABLE 8 *Eff ct of Passing Normal Thymocytes through Ig Anti-Ig Columns on Their Ability to General ADCC Activity in ATCs* b)

Group no.	800 R hosts	Donor thymocytes	Passed Ig anti-Ig column	Per cent specific 51Cr release from) Ab-CRBC 1 1	1:5	1.25	P815 1 1	1 10
I	Balb/cxCBA	—		0.9	8.7	21.5	—	—
IIa		Balb/cxCBA	—	3.9	15 7	30.8	0 8	0.7
IIb			+	4 2	20.8	41.5	1.2	3.2
IIIa		CBA	—	2.8	9 7	23.1	1.5	47.5
IIIb			+	5 7	23.8	42.1	30.8	58.5

) Spontaneous release Ab-CRBC = 3.5 per cent, P815 = 8.1 per cent. 1 1 1.5, see Table 7
b) Effector cells were harvested 6 day after irradiation and injection, when they had the following characteristics

Assay	Group no. I	IIa	IIb	IIIa	IIIb
% Θ pos cells	61.3	79.3	81.8	85.9	92.9
% EA-RFC	20 8	33 8	37.9	51 7	58.9
% EAC-RFC	5.9	4.3	1.8	5.9	0.7

serum and complement technique. Two types of effector cells were produced 1) CBA anti-Balb/cxCBA ATCs and 2) Balb/cxCBA anti-CBA ATCs. Treatment of these two types of ATCs with CBA anti-Balb/c antiserum and complement would kill host-derived cells in

TABLE 5 *Effect of Removing Adherent and/or Phagocytic Cells from ATCs on Their Cytotoxic Potential against Ab-CRBC and P815 Tumour Cells*

Effector cells a)	Fractionation technique b)	Per cent specific ^{51}Cr release from c) Ab-CRBC 1 1	1 5	1.25	P815 1 1	1 10
800 R Balb/cxCBA	C	—	11.8	—	—	—
–"–	Fa+Fe	—	5 1	—	—	—
(Balb/cxCBA) anti Balb/cxCBA ATCs	C	4 2	12.5	20.5	—	—
–"–	Fa	3 6	5.3	11 4	—	—
–"–	Fe	2 9	6 1	12.8	—	—
– –	Fa+Fe	3.6	3 7	11.9	—	—
CBA anti Balb/cxCBA ATCs	C	6.8	17 6	24 1	18.7	34 8
–"–	Fa	1 9	4.2	14.9	20.3	57.9
– –	Fe	2 1	5.7	13.2	22.9	53.6
–"–	Fa+Fe	0.9	3.8	6.2	30 1	62.4

a) Spleen cells from 800 R irradiated Balb/cxCBA mice either not reconstituted or injected with 10⁸ syngeneic thymocytes or injected with 10⁸ CBA thymocytes.

b) C = cells not treated (incubated in a plastic tube at 37 C) Fa = cells not adherent to a plastic surface. Fe = cells not removed after treatment with carbonyl-iron and magnet. Fa+Fe = cells which are recovered after incubation on to a plastic surface with carbonyl iron.

c) 1 1 1 5 = target effector cell ratio Spontaneous release Ab-CRBC = 3 4 per cent, P816 = 8 3 per cent, – = not done.

TABLE 6 *Combined Treatment with Alloantiserum and Adherence Fractionation. Impact on the Cytotoxic Potential of ATCs)*

Fractionation technique b)	Target Effector Cell ratio	Effector cells b) CBA anti Balb/cxCBA ATCs C d)	anti-Balb/c d)	(Balb/cxCBA) anti-CBA ATCs C	anti-Balb/c
C	1 20	21 9	10 7	28.5	13.9
	1 4	9.3	3 1	12.8	3.8
Fa+Fe	1 20	11 8	12 1	13 1	1 2
	1 4	4 2	3.9	2.7	0.9

a) Results are expressed as per cent specific ^{51}Cr release from Ab-CRBC/ Spontaneous release = 1.8 per cent.

b) See Table 5

c) Spleen cells from 800 R Balb/cxCBA mice injected with 10⁸ CBA thymocytes (I) and 800 R CBA mice injected with Balb/cxCBA thymocytes (II) Per cent θ positive cells I = 91 6 II = 93.4 Per cent EA RFC I = 47.3 II = 48.1

d) C = cells treated with complement, anti Balb/c = cells treated with anti-Balb/c antiserum and complement. For further details, see Table 1

sor T cells are FcR (2, 10 21) and that they would seem to have the same Ly antigens on their surface as CTL have (= Ly 2,3 (2)) Experiments where FcR and FcR precursor CTL are mixed have produced evidence that FcR CTL may indeed suppress the generation of CTL from FcR precursor CTL (25) Furthermore, Con A may induce suppressor T cells of the Ly-2,3 type (2) Whether or not the Con A responding cells among ATCs or among normal thymocytes which can be adsorbed on to Ig anti-Ig columns (i.e. are FcR (11 23)) act as suppressor cells (Table 8) is at present under investigation.

However one question is still unsolved How do the FcR thymocytes become FcR? Are they triggered in the irradiated mouse environment to synthesize and express FcR, or do they acquire their FcR passively? We have previously indicated that sera from irradiated mice could "arm FcR thymocytes and spleen cells to become FcR (see 22) We still find some sera with this capacity but in no instance were the armed" FcR T cells cytotoxic against Ab-CRBC Furthermore, if FcR T cells are induced into CTL *in vitro* via a mixed lymphocyte reaction, the CTL are FcR and no cells with ADCC activity emerge (12, 25) Since most of the adherent ADCC cells from the irradiated host mice are FcR one might wonder whether FcR thymocytes could be armed" with FcR from the FcR macrophages. Indication of such a possibility has been published (29) and more *in vitro* "arming experiments with FcR macrophages mixed with Ig anti-Ig column passed thymus or spleen cells should solve this problem.

Note added in proof

Further experiments have shown that the donor-derived effector T cells do also kill antibody coated K 562 cells (a human *in vitro* B cell line)

This work was supported partly by the *Danish Medical Research Council* (J no 512 2642 and 512-3506) The skilful technical assistance of Miss *J Lieberkind* and Miss *B R* ... is gratefully acknowledged. The authors wish to thank Drs. *P Golstein A K Kimura* and *A. B* ... for helpful discussions, and for reading the manuscript.

REFERENCES

1 *Blomgren H.*, Studies on the proliferation of thymus cells injected into syngeneic or allogeneic irradiated mice. Clin. exp. Immunol. *8* 279–283 1971

2. *Cantor H., Shen, F W & Boyse E. A* Separation of helper T cells from suppressor T cells expressing different Ly components. II. Activation by antigen after immunization, antigen specific suppressor and helper activity are mediated by distinct T cell subclasses. J Exp. Med. *143* 1391–1401 1976.

3 *Dennert G. & Lennox E. S.*, Phagocytic cells as effectors in cell-mediated immune system. J Immunol. *111* 1844–1849 1973

4 *Forman, J., Britton S & Möller G.*, Ability of normal and activated thymus derived cells to act as cytotoxic effector cells against antibody coated targets (Unpublished observations, 1974)

5 *Gale R. P & Zighelboim J* Polymorphonuclear leucocytes in antibody-dependent cellular cytotoxicity J Immunol. *114* 1047–1051 1975

6. *Golstein P., Wigzell, H., Blomgren H & Svedmyr E A. J.*: Cells mediating in vitro cytotoxicity II Probable autonomy of thymus-processed lymphocytes (T cells) for the killing of allogeneic target cells. J Exp. Med. *135* 890–904, 1972.

7 *Golstein, P., S chirrmacher V Rubin B. & Wigzell H* Cytotoxic immune cells with specificity for defined soluble antigens. II. Chasing the killing cells. Cell. Immunol. *9* 211–224 1973

8. *Greenberg, A H Chen L., Walker L., A van Villena, A. & Roitt I M* Characteristics of the effector cells mediating cytotoxicity against antibody coated target cells. II. The mouse non-adherent K cell. Eur J Immunol. *5* 474–482, 1975

9 *Hertel-Wulff B. & Rubin, B* Lymphocyte proliferation in vitro induced by soluble protein antigens. II. Cellular requirements. Eur. J Immunol. *6* 418–424 1976.

10. *Herzenberg, L. A., Okumura, K., Cantor H Sato U T Shen, F W., Boyse E. A. & Herzenberg, L. A.*, T cell regulation of antibody responses demonstration of allotype specific helper T cells and their specific removal by suppressor T cells. J Exp. Med. *144* 330–341 1976.

11 *Haier-Madsen, M & Rubin, B.*, Mitogenic responses of mouse thymus and spleen cells. Effect of Immunoadsorbent procedures. Acta path. microbiol. scand. Sect. C, *83* 429–438, 1975

12. *Kimura, A. K Rubin B. & Andersson, L. C.*: Evidence for "K" cell killing by F receptor

the former case and donor-derived cells in the latter case. Thus with type 1) cells removal of adherent cells in addition to the removal of host type cells should not give any further reduction in ADCC, whereas with type 2) cells such treatment should completely abrogate ADCC. The results in Table 6 show that ADCC activity among ATCs is performed by two types of effector cells, donor-derived T cells and host-derived adherent cells.

Fractionation Characteristics of ADCC Effector Cells on Ig Anti Ig Columns

Almost per definition ADCC effector cells express FcR on their membrane. It was therefore of interest to see whether both types of effector cells would be adsorbed on to Ig anti Ig columns. The results in Table 7 show that both types of ADCC effector cells were adsorbed on to the columns, even in the presence of EDTA. This means that FcR T cells are adsorbed on to Ig anti Ig columns (24) and the results indicate that EDTA does not inhibit the adsorption of FcR T cells among ATCs.

Finally since the donor-derived T cells with ADCC activity originate from the thymocytes which contain about 2–5 per cent EA RFC (20 22) some of which are endowed with ADCC activity (26) we tested whether the T cells showing ADCC activity were derived from the FcR cells among thymocytes. The results in Table 8 show that both ADCC and T cell mediated cytotoxicity of ATCs derived from Ig anti Ig column passed thymocytes were significantly enhanced as compared to the situation where the ATCs were derived from non passed thymocytes. This would indicate that suppressor cells among thymocytes (19) are retained on the column and that FcR T cells among ATCs are not the offspring of FcR thymocytes.

DISCUSSION

The present experiments were designed to identify the effector cells of ADCC among ATCs. The results show that there were at least two types of effector cells, host-derived adherent cells and donor-derived T cells (Table 6) Furthermore, the donor-derived T cells with ADCC activity would seem to originate from FcR thymocytes (Table 8) Thus, like other T cell functions which can be induced in lethally irradiated mice (helper T cell activity cytotoxic T cell activity and PHA responsiveness (6 12, 13 22, 23, 28)) the FcR T cells mediating ADCC can be induced from apparently FcR precursors. However helper T cells would seem to stay FcR⁻ throughout their life-span (22 30) whereas cytotoxic precursor T cells may develop into both FcR CTL and FcR CTL (12 13 24 25) It is still an open question whether these two types of CTL are two independent T cell lines, or whether the FcR T cells develop from FcR T cells (see discussion in 24) In addition, we do not know whether the FcR T cells with ADCC activity are the same cells as the FcR CTL (see discussion in 12) Recent observations have added to the complexity of the problem by showing that FcR T cells may kill target cells coated with either IgM or IgG (14 18)

The problem from our previous publications on this subject (22 23) the function of FcR T cells among thymocytes, has been solved in this and a parallel study (12) They can display cytotoxicity against both specific allogeneic target cells and antibody-coated "unspecific target cells. These findings were based on the separation of FcR T cells from FcR⁻ T cells on $1 \times g$ sedimentation gradients (12 13) However we found quite frequently that Ig anti Ig column passed ATCs displayed enhanced cytotoxicity against allogeneic target cells as compared to non-passed ATCs (20) It was concluded from such results that most CTL among ATCs were FcR a conclusion which was supported by the fact that EDTA would not elute FcR T cells among ATCs from Ig anti Ig columns (*Rubin* unpublished) This could be done with FcR T cells from normally immunized mice (24) One possible explanation for these different results may be that FcR CTL suspensions contain (or are themselves) suppressor cells. It has been demonstrated that suppres-

Acta path. microbiol. scand. Sect. C, 85 277-283 1977

IN VITRO ACTIVATING PROPERTIES OF POLYENE ANTIBIOTICS FOR MURINE LYMPHOCYTES

L. HAMMARSTRÖM and C. I. E. SMITH

Division of Immunobiology Wallenberg Laboratory Karolinska Institute
Lilla Frescati, Stockholm, Sweden

Hammarstrom, L. & Smith, C. I. E. In vitro activating properties of polyene antibiotics for murine lymphocytes. Acta path. microbiol. scand. Sect C, 85 277-283 1977

The effect of four polyene antibiotics, Candicidin, Etruscomycin, Filipin and Pimaricin upon mouse lymphocytes was studied. Polyene antibiotics are known to form aqueous pores in the cell membranes inducing known anion or cation selective fluxes. Candicidin was capable of inducing marked DNA-synthesis and polyclonal antibody production when added to normal spleen cells. Etruscomycin and Pimaricin showed a weak inconsistent DNA synthetic stimulatory effect, whereas Filipin was found to be totally uneffective. The stimulating property of Candicidin was also demonstrated on spleen cells from nude mice whereas there was no effect on cortisone resistant thymocytes or spleen cells passed through a nylon fibre column. Thus we conclude that Candicidin is a PBA for mouse lymphocytes. We have previously reported that the two anionselective polyenes, Nystatin and Amphotericin B are polyclonal B-cell activators for mouse lymphocytes and in this paper the possible mechanism of triggering is further discussed.

Key words: Polyene antibiotics mitogens polyclonal B-cell activators.

L. Hammarström, Division of Immunobiology Wallenberg Laboratory Lilla Frescati, S-104 05 Stockholm, Sweden.

Received 27.iii.76 Accepted 14.iii.77

A large number of polyene antibiotics produced by actinomycetes have been isolated and described. Depending upon the number of conjugated carbon-to-carbon double bonds that are present in the molecule, the polyenes can be divided into four groups, tetraenes, pentaenes, hexaenes and heptaenes (for review see 16). The biological activity of these antifungal drugs seems to be dependent on the ability to form aqueous pores in cell membranes, and detailed models for polyene membrane interactions have been proposed (1 4 7 11). Certain polyene antibiotics such as Nystatin and Amphotericin B have been found to be preferably anion-selective (4) whereas others, such as Candicidin, have been found to increase mainly the permeability for cations (15). The reason for this charge-dependent ion-selectivity is as yet not known.

We have previously reported that the two anion-selective pore inducing polyenes, Nystatin and Amphotericin B, activate resting bursa-derived lymphocytes (B-cells) to proliferation and polyclonal antibody synthesis in the mouse (12). Chemical compounds with these activating properties are referred to as poly

bearing alloactivated cytotoxic T lymphocytes. In press. Scand. J Immunol *6* 1977

13 *Kramer P H Elliott B E. & von Boehmer H* Fc rosetting and nonrosetting T cells are killer cells. Eur J Immunol. *6* 138–139 1976.

14 *Lamon E W Whitten H D Skurzak H M Andersson B & Lidin B* IgM antibody dependent cell mediated cytotoxicity in the moloney sarcoma virus system The involvement of T and B lymphocytes as effector cells. J Immunol *115* 1288–1294 1975

15 *Mackmore A I Nelson D L. & Blaese R M* The cytotoxic effector potential of some common non lymphoid tumors and cultured cell lines with phytohemagglutinin and heterologous anti-erythrocyte antisera. J Immunol. *114* 1001–1007 1975

16 *Parish C R Kirov S M Bowern N & Blanden R V* A one step procedure for separating mouse T and B lymphocytes. Eur J Immunol. *4* 808–813 1974

17 *Perlmann P Wigzell H Golstein P Lamon E. W Larsson A., O'Toole C Perlmann H & Svedmyr E. A J* Cell mediated cytotoxicity in vitro Analysis of active lymphocyte subpopulations in different experimental systems. Adv Biosciences *12* 71–82 1974 Pergamon Press.

18 *Perlmann P* Personal communication 1976

19 *Rubin B* Studies on the adsorbability of graft-versus-host reactive lymphocytes. Clin. exp. Immunol *20* 513–525 1975

20 *Rubin B* Autonomy in the generation of specific T lymphocyte functions In *Talwar G P* (Ed.) Regulation of growth and differentiated function in eukaryote cells. Raven Press, N Y 1975 p 249–261

21 *Rubin B* Regulation of helper cell activity by specifically adsorbable T lymphocytes. J Immunol. *116* 80–85 1976

22. *Rubin B & Hertel Wulff B* Biological significance of Fc receptor-bearing cells among activated T lymphocytes Scand. J Immunol. *4* 451–462, 1975

23 *Rubin B Hertel Wulff B & Hoier Madsen M* A study on the immunological function of Fc receptor-bearing cells among activated thymocytes. In *Feldmann M & Globerson A.* (Eds.) Immune reactivity of lymphocytes. Plenum Press N Y 1976 p 361–367

24 *Rubin B Jorgensen P N Geisler F & Hoier Madsen M* Impact of Ig anti-Ig column fractionation on T lymphocyte functions. A study of subpopulations eluted with EDTA. Scand. J Immunol. *5* 345–359 1976

25 *Rubin B & Hertel Wulff B.* T lymphocyte recognition of alloantigen in vitro II Significance of Fc receptor positive and negative responder T cells in the generation of cytotoxic T lymphocytes from normal or immune mice. Unpublished results.

26. *Rubin B Hoier Madsen M & Ropke C.* In vitro analysis of bone marrow precursor B or T lymphocytes. A correlation to in vivo studies. Unpublished results.

27 *Shearer G M., Garbarino C A & Cudkowicz G* In vitro induction of F 1 hybrid anti-parent cell-mediated cytotoxicity J Immunol. *117* 754–759 1976.

28. *Sprent J & Miller J F A. P.* Activation of thymus cells by histocompatibility antigens. Nature New Biol. *234* 195–199 1971

29 *Saal J G., Rieber E.P Hadam M & Rieth-muller G* Lymphocytes with T-cell markers cooperate with IgG-antibodies in the lysis of human tumor cells. Nature *265* 158–160, 1977

30 *Stout R & Herzenberg L. A* The Fc receptor on thymus-derived lymphocytes. I Detection of a subpopulation of murine T lymphocytes bearing the Fc receptor J Exp. Med. *142* 611–621 1975

31 *von Boehmer H & Shortman K.* The separation of different cell classes from lymphoid organs. IX. A simple and rapid method for removal of damaged cells from lymphoid cell suspensions. J Immunol Meth. 2 293–298, 1973

32. *Walker W S & Demus A* Antibody-dependent cytolysis of chicken erythrocytes by an in vitro-established line of mouse peritoneal macrophages. J Immunol. *114* 1047–1051 1975

TABLE 1 *Induction of DNA Synthesis by Mitogens in Normal and Column Purified Spleen Cells from CBA Mice (5 × 10⁵ Cells/Culture) on Day 2 Results Are Mean of Triplicate Cultures in 2 Experiments (±1 SD)*

Mitogen	Optimal dose µg/ml	Normal spleen cells	Column purified spleen cells
Amphotericin B	10	25703 ± 1571	1301 ± 76
Nystatin	10	21888 ± 2112	1253 ± 167
Candicidin	10^{-1}	23208 ± 1930	1402 ± 90
Etruscomycin	10^{-}	8118 ± 728	1372 ± 111
Pimaricin	10	7507 ± 913	1351 ± 98
Phytohaemagglutinin	1	74320 ± 3407	82598 ± 3392
Lipopolysaccharide	100	60227 ± 5339	1515 ± 207
0	–	6891 ± 528	1225 ± 152

biotics, which all were preservative free, were dissolved in dimethylsulfoxide as stock solution of 10 mg/ml and thereafter diluted in ordinary culture medium.

Mitogens Lipopolysaccharide from *E coli* O55 B5 (LPS) was prepared by phenol-water extraction by Prof. T *Holme* (Dept. Bacteriology Karolinska Institute, Stockholm, Sweden) Phytohaemagglutinin (PHA) was purchased from Wellcome Reagents Ltd, Great Britain.

RESULTS

Kinetics

Spleen cells from C57BL or A.CA mice were cultured with optimal amounts of the antibiotics. ³H thymidine was added on day

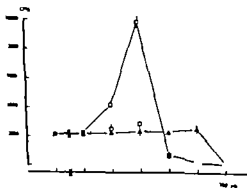


Fig 3 Induction of DNA-synthesis by candicidin (□) etruscomycin (□) or pimaricin (Δ –) or spleen cells from nude mice or BALB/ background on day 2. Results are mean of triplicate serumfree cultures (5 × 10⁵ cells/culture) Vertical bars represent 1 SD

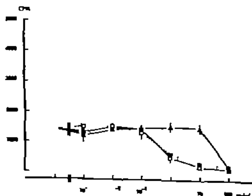


Fig 4 Induction of DNA-synthesis by candicidin (□ –) etruscomycin (□) or pimaricin (Δ –) or cortisone-resistant thymocytes from CBA mice on day 2 Results are mean of triplicate serumfree cultures (5 × 10⁵ cells/culture) Vertical bars represent 1 SD

0 1 2 or 3 and the cultures were harvested 24 h later Candicidin induced a DNA synthetic peak response four to six times background on day 2, Etruscomycin and Pimaricin gave a very weak, inconsistent stimulation with maximal values less than twice background. Filipin never induced an increased DNA-synthesis (Fig 1)

Dose Response

Spleen cells from A, C57BL, Balb/c and congenitally athymic "nude" mice on Balb/c background, A.CA, CBA and C3H/HeJ were cultured with various concentrations of the polyenes. DNA-synthesis was measured on

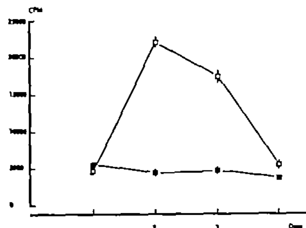


Fig. 1 Kinetics of the induction of DNA-synthesis in serumfree cultures with spleen cells from A.CA mice (5×10^5 cells/culture) by 0.1 μg/ml of candicidin (□–) Background values (■–) Results are mean of triplicate cultures. Vertical bars represent 1 SD

clonal B-cell activators (PBA) In order to further study the basic mechanisms of B-cell triggering, we have now extended the work to include some other chemically well characterized polyenes to test for PBA properties

Increased permeability to divalent cations has previously been reported to be essential in lymphocyte triggering (9 17) and the polyenes could serve as an experimental tool to test the validity of this hypothesis.

In this paper we present data on the effect of a pore-inducing anionselective tetraene Etruscomycin a non pore inducing tetraene, Pimaricin, a membrane-disrupting pentaene Filipin and a pore-inducing cation selective heptaene Candicidin.

MATERIALS AND METHODS

Mice A (H 2a) C57BL(H 2b) Balb/c (H 2d) and congenitally athymic "nude mice on Balb/c background A.CA (H 2f) CBA(H 2k) and C3H/HeJ (H 2k) mice of both sexes were used at the age of 4–8 weeks.

Preparation of purified T and B-cell populations. T cells were prepared in two ways 1 thymocytes from mice given 4 mg of cortisone acetate intraperitoneally 48 h before removal of the thymus or 2. spleen cells passed through a nylon fiber column (14) Pure B cells were obtained from spleens of nude mice.

Culture conditions Cells were cultured in serum-free Mishell-Dutton medium (19) For measurements of DNA synthesis, cells were cultured in microplates. Each well contained 5×10^5 cells in 0.20 ml. For estimation of antibody-secreting cells, cultures were set up in plastic Petri dishes containing 10^7 untreated or iron-treated spleen cells in 1 ml. All cultures were kept on a rocking platform at 37 C in an atmosphere of 10 per cent CO_2, 83 per cent N_2 and 7 per cent O_2 (19)

Assay of DNA synthesis After 0–3 days of incubation ^{3}H thymidine (1 μCi/culture) (Radiochemical Centre, Amersham, England) was added. 24 h later the cultures were harvested (multiple sample harvester A/S Skatron, Lierbyen, Norway) washed with distilled water and collected on glass fibre filters. All filters were dried overnight and transferred to scintillation vials. 2 ml of toluene-based scintillation fluid was added and radioactivity measured in a scintillation spectrophotometer (Tri-Carb, Packard)

Assay of antibody-secreting cells Sheep erythrocytes (SRBC) stored in Alsever's solution and washed three times in balanced salt solution before use were heavily coupled with NNP (4-hydroxy-3,5-dinitro-phenacetyl) (21) or FITC (20) Assays for detection of plaque-forming cells were performed with a modification of the local hemolysis-in-gel assay (13) as described by *Bullock & Möller* (5)

Polyenes Candicidin was a gift from Gallia AB, Stockholm, Sweden. Etruscomycin (Lucensomycin®) was kindly supplied by Farmitalia, Milano, Italy Filipin was a gift from Upjohn Company Kalamazoo, Michigan, USA and Pimaricin was kindly supplied by Cyanamid Svenska AB, Stockholm, Sweden. Amphotericin B and Nystatin were gifts from Squibb AB, Lidingö Sweden. The anti-

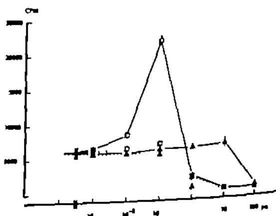


Fig. 2 Induction of DNA-synthesis by candicidin (□–) etruscomycin (□) filipin (△) or pimaricin (▲–) in serumfree cultures with spleen cells from CBA mice (5×10^5 cells/culture) on day 2. Results are mean of triplicate cultures. Vertical bars represent 1 SD

cally significant. Filipin never induced any plaque forming cells. Only IgM plaque-forming cells were induced.

Effect of Adherent Cells

The stimulatory ratio in spleen cells treated with carbonyl iron powder to remove macrophages (18) showed a slight increase for the DNA synthetic response. The stimulatory ratio was also increased for the formation of plaque-forming cells after the depletion of adherent cells (Table 3)

DISCUSSION

In this paper we describe the polyclonal B-cell activating properties of the polyene antibiotic Candicidin. Three other polyenes Filipin, Etruscomycin and Pimaricin showed no marked mitogenic effect. The cells affected were B-cells, since an increased DNA synthesis was seen in purified B-cell suspensions contrary to purified T-cell suspensions. In parallell with the increased DNA-synthesis, an induction of polyclonal antibody producing cells is seen which is not dependent upon macrophages since removal of adherent cells does not decrease the mitogenic activity

Previously we have presented data showing that the tetraene Nystatin and the heptaene Amphotericin B are polyclonal B-cell activators (PBA) for murine spleen cells (12). These two antifungal antibiotics are known to induce pores in membranes that are preferably anion-selective (4). In order to explain the PBA activity of these two polyenes, various theories were proposed. One possible mechanism for immunocyte triggering is that ionic fluxes across the cell membrane are primarily involved. This has been claimed to explain the mitogenic properties of the ionophore A 23 187 which is thought to transfer divalent cations, such as calcium and magnesium, across cell membranes (17) an effect, which however has been questioned (8). Another possible mechanism, that may be the cause of lymphocyte triggering is that a disturbed cellular ionic balance or decreased membrane stability in itself may stimulate cell proliferation and induction of effector functions. This way of activation has been proposed for mitogens such as LPS and phytohaemagglutinin (7 9 10). A third possibility is that binding of a mitogen to a specific receptor not identical to the Ig receptor (5) will result in an activating signal to the lymphocyte.

To test these possible mechanisms of triggering we chose to study other well characterized polyenes. The anion-selective pore inducing tetraene, Etruscomycin, a nonpore inducing tetraene Pimaricin, a membrane disrupting pentaene, Filipin, and a cation-selective pore-inducing heptaene, Candicidin. As shown in this paper the cation-selective polyene Candicidin was as good as the two anion-selective polyenes Nystatin and Amphotericin B in inducing DNA-synthesis and the formation of plaque forming cells in murine spleen cell cultures (12). However the selectivity for cations and anions is not absolute, and small amounts of anions may pass through the Candicidin induced cation-selective pores and vice versa. Filipin, which is known to cause disruption of cell membranes with consequent leakage of large proteins such as enzymes (6) had no lymphocyte stimulatory effect, only being toxic at high concentrations. Etruscomycin and Pimaricin were weakly if at all stimulatory for mouse B-cells. Etruscomycin is anion-selective as Nystatin and Amphotericin B (7) whereas Pimaricin due to its short hydrophobic tail does not induce pores (7)

A certain ion-selectivity for B cell triggering by polyenes does therefore not seem to be an absolute requirement, since both anion-selective and cation-selective antibiotics are PBA. Pore formation, and thus a disturbed cellular ionic balance could be a necessary requirement for B cell activation, since a non-pore inducing polyene was not mitogenic and whereas three out of four pore-inducing polyenes were mitogenic. The weak effect of Etruscomycin, in spite of the well documented ability of this polyene to induce pores, could be due to the strong temperature de-

TABLE 2 *Induction of Antibody Synthesis by Candicidin 10⁷ Normal Spleen Cells from A.CA Mice Were Cultured Serumfree in 1 ml Petri Dish and Assayed for Plaque-forming Cells on Day 2 Results Are Means of Two Experiments (± 1 SD)*

Dose in µg/ml	IgM anti-SRBC PFC/10⁶ cells	IgM anti-NNP PFC/10⁶ cells	IgM anti-FITC PFC/10⁶ cells
10	0 ± 0	0 ± 0	0 ± 0
1	1 ± 1	2 ± 1	3 ± 2
10^{-1}	22 ± 3	119 ± 28	134 ± 33
10^{-2}	13 ± 4	47 ± 9	61 ± 15
10^{-3}	6 ± 3	23 ± 5	31 ± 6
0	5 ± 4	21 ± 6	25 ± 4

TABLE 3 *Induction of Anti-NNP Antibodies by Different Mitogens. 10⁷ Spleen Cells, Irontreated or Normal from CBA Mice Were Cultured Serumfree in 1 ml Petri Dish and Assayed on Day 2. Results Are Mean of 2 Experiments (± 1 SD)*

Mitogen	Dose µg/ml	IgM anti NNP PFC/10⁶ normal spleen cells	IgM anti-NNP PFC/10⁶ irontreated spleen cells
Nystatin	10	1?4 ± 11	1?7 ± 9
Candicidin	10^{-1}	117 ± 15	109 ± 12
LPS	100	432 ± 37	456 ± 31
O	0	21 ± 6	16 ± 4

day 2 Optimal response was obtained using a concentration of 0 1 µg/ml of Candicidin Etruscomycin showed a weak effect at 0 1 µg/ml and Pimaricin at 10 µg/ml Filipin never induced an increased DNA synthesis at any dose (Fig 2) No consistant variation between the various mouse strains tested was found (data not shown)

Effect on T and B Cells

Purified T cells were obtained from the thymus of cortisone treated mice or from spleen cells passed through a nylon fibre column. The source of B cells were spleens of athymic 'nude' mice. The cell preparations were cultured in microplates with various amounts of polyenes added and DNA synthesis was measured on day 2 The results given in Table 1 and Figs. 3 and 4 indicate that Candicidin, as well as the previously tested anion selective polyenes, selectively activate B-cells. The 'purity' of the cell suspensions used was tested by including controls stimulated by phytohaemagglutinin (PHA) and lipopolysaccharide (LPS) respectively Cortisone resistant thymus cells or cells passed through a nylon fibre column showed almost no response to LPS (stimulation index less than 2) whereas the PHA response was slightly enhanced (Table 1)

Induction of Polyclonal Antibody Synthesis

B-cell mitogens induce polyclonal antibody synthesis, which can be measured in the plaque assay with SRBC coupled with various haptens or antigens. Spleen cells were assayed after 2 days of culture in Petri dishes containing various concentrations of Candicidin Etruscomycin, Filipin and Pimaricin. The results summarized in Table 2 correspond well with the effect of Candicidin on DNA-synthesis, with "maximal" plaque values amounting to four to six times background. Etruscomycin and Pimaricin sometimes induced a minor increase in plaque-forming cell numbers wh ch was not statisti-

of dissociated spleen cell cultures from normal mice J Exp. Med. *126* 423–442 1967

20. *Möller G* Effect of B cell mitogens on lymphocyte subpopulations possessing C'3 and Fc receptors. J Exp. Med. *139* 969–982, 1974

21 *Pasanen, V J & Mäkelä O* Effect of the number of haptens coupled to each erythrocyte on haemolytic plaque formation. Immunology *16* 399–407 1969

22 *Shinitzky M & Inbar M*. Microviscosity parameters and protein mobility in biological membranes. Biochim. Biophys. Acta (Amsterdam) *433* 133–149 1976.

pendence of this phenomenon, since Etruscomycin is known to affect membranes much more effectively at 0° C than at room temperature (7)

Polyenes are known to bind to cholesterol in mammalian cell membranes. It therefore seems likely, that this sterol or some other membrane constituent, the function of which is dependent upon the behaviour of cholesterol could be directly involved in the triggering process of B-cells by polyene antibiotics. Whether this would nonspecifically activate the cells only via an increased membrane fluidity due to the complexing with cholesterol which has known regulatory functions in the cell membrane (22) or due to binding to a specific mitogen receptor containing cholesterol, with subsequent triggering is at present not known. To elucidate these different alternatives, experiments with other cholesterol binding substances must be performed.

The technical assistance of Ms. *Inger Cederberg* is gratefully acknowledged. This work was supported by grants from the *Swedish Cancer Society* and *Torsten and Ragnar Söderberg's Foundation*

REFERENCES

1 *Andreoli T E* On the anatomy of Amphotericin B-cholesterol pores in lipid bilayer membranes. Kidney Int. *4* 337–345 1973

2 *Barnett R E. Scott R E Furcht L. T & Kersey J H* Evidence that mitogenic lectins induce changes in lymphocyte membrane fluidity Nature (London) *249* 465–466 1974

3 *Bullock W W & Möller E* Spontaneous B cell activation due to loss of normal mouse serum suppressors Eur J Immunol. *2* 514–517 1972

4 *Cass A Finkelstein A & Krespi V* The ion permeability induced in thin lipid membranes by the polyene antibiotics nystatin and amphotericin B. J Gen. Physiol. *56* 100–124 1970

5 *Coutinho A & Möller G* Immune activation of B cells Evidence for "one nonspecific triggering signal" not delivered by the Ig receptors. Scand. J Immunol. *3* 133–146 1974

6 *De Kruijff B., Gerritsen W J., Oerlemans, A Van Dijck P W M., Demel, R. A. & Van Deenen L. L. M.* Polyene antibiotic sterol interactions in membranes of Acholeplasma Laidlawii cells and lecithin liposomes. Biochim. Biophys. Acta (Amsterdam) *339* 30–43 1974

7 *De Kruijff B. & Demel R. A* Polyene antibiotic-sterol interactions in membranes of *Acholeplasma laidlawii* cells and lecithin liposomes. III Molecular structure of the polyene antibiotic-cholesterol complexes. Biochim. Biophys. Acta (Amsterdam) *339* 57–70, 1974

8. *Devore D I & Nastuk W L.* Effects of calcium ionophore X537 on a frog skeletal muscle. Nature (London) *253* 644–646, 1975.

9 *Diamantstein T & Ulmer A.* The control of immune response in vitro by Ca²⁺ II. The Ca⁺⁺ dependent period during mitogenic stimulation. Immunology *28* 121–125, 1975.

10. *Ferber E., Reilly C E., De Pasquale G & Resch K.* Lymphocyte stimulation by mitogens Increase in membrane fluidity caused by changes of fatty acid moities of phospholipids. In Lymphocyte Recognition and Effector Mechanisms. (Eds.) *Lindahl Kiessling, K. and Osoba, D* Acad. Press. New York 1974 pp. 529–534

11 *Finkelstein A. & Holtz R.* Aqueous pores created in thin lipid membranes by the polyene antibiotics nystatin and amphotericin B. Membranes *2* 377–408, 1973

12. *Hammarström L. & Smith E.* Mitogenic properties of polyene antibiotics for murine B cells. Scand. J Immunol. *5* 37–43 1976.

13 *Jerne N K & Nordin A A.* Plaque forming in agar by single antibody producing cells. Science *140* 405–406, 1963

14 *Julius M H Simpson E. & Herzenberg, L. A* A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur J Immunol. *3* 645–649 1973

15 *Karamov K. M & Liberman Y A* Ionic permeability of bimolecular membranes in the presence of polyene antibiotics. II Levorin, Trichomycin, Candicidin. Biofizika *18* 271 279 1972.

16 *Kinsky S C* Antibiotic interactions with model membranes. Ann. Rev Pharmacol. *10* 119–142, 1970

17 *Luckasen J R White J G & Kersey J H* Mitogenic properties of a calcium ionophore A23187 Proc. Nat. Acad. Sci. *71* 5088–5090, 1974

18. *Lundgren G., Zukoski C H F & Möller G* Differential effect of human granulocytes and lymphocytes on human fibroblasts in vitro. Clin. Exp Immunol. *3* 817–836, 1968.

19 *Mishell R I & Dutton R W.* Immunization

of dissociated spleen cell cultures from normal mice. J Exp. Med. *126* 423–442, 1967
20. *Möller G* Effect of B cell mitogens on lymphocyte subpopulations possessing C'3 and Fc receptors. J Exp. Med. *139* 969–982, 1974
21. *Pasanen V J & Mäkelä, O* Effect of the number of haptens coupled to each erythrocyte on haemolytic plaque formation. Immunology *16* 399–407 1969
22. *Shinitzky M & I bar M.*, Microviscosity parameters and protein mobility in biological membranes. Biochim Biophys. Acta (Amsterdam) *433:* 133–149 1976.

Acta path. microbiol. scand Sect. C 85 284–288 1977

INFLUENCE OF HYDROCORTISONE ON GRANULOCYTE FUNCTION AND GLUCOSE METABOLISM

KAREN ELINA CHRISTIE, BERIT KJOSEN and CLAUS O SOLBERG

Medical Dept. B, University of Bergen, Bergen, Norway

Christie K. E., Kjosen, B & Solberg, C. O Influence of hydrocortisone on granulocyte function and glucose metabolism. Acta path microbiol. scand. Sect. C, 85 284–288 1977

Examination has been made of the influence of hydrocortisone on the *in vitro* phagocytosis and intracellular killing of *Staphylococcus aureus* by human neutrophils and the production of lactate and CO_2 during phagocytosis of latex particles. In high concentrations, 0.5–2 mg per ml hydrocortisone caused a significant reduction in the phagocytosis and the production of lactate. Neither the bactericidal activity nor the production of $^{14}CO_2$ from (U-^{14}C)glucose in phagocytizing leukocytes was influenced by these hydrocortisone concentrations.

Key words glucose metabolism granulocytes hydrocortisone intracellular killing phagocytosis.

C. O Solberg Medical Department B N 5016 Haukeland sykehus, Norway

Received 15.ii.77 Accepted 18.iii.77

Evidence is now accumulating that commonly used antiinflammatory drugs may influence granulocyte function (13) In high concentrations, glucocorticosteroids inhibit some of the biochemical events triggered in granulocytes by ingestion of bacteria (6 10 11) However the importance of these observations for the phagocytosis and intracellular killing of bacteria has not been definitely settled (1 7 14 22) and the results are sometimes conflicting (11 14) This may be due in part to difficulties in methodology particularly in the separation of extracellular and intracellular bacteria in an *in vitro* phagocytic system.

In the present study the influence of hydrocortisone on the phagocytic and bactericidal activities of human neutrophils has been examined using a recently developed method which facilitates a precise *in vitro* evaluation of each function (17 18) As the phagocytic and bactericidal activities are dependent on certain metabolic processes in the leukocytes, the influence of hydrocortisone on the leukocyte glucose metabolism, as expressed by the production of CO_2 and lactate has been examined and compared with the results of the granulocyte-bacteria experiments.

MATERIAL AND METHODS

Leukocytes

Leukocytes were obtained from healthy human donors by Isopaque/dextran sedimentation of heparinized blood as described previously (17) The cells were suspended in Hanks balanced salt solution (HBSS) containing 1 per cent gelatin. Eighty-four to 90 per cent of the leukocytes were neutrophil granulocytes and 96 to 99 per cent of the neutrophils resisted staining with trypan blue and retained their functional integrity intact as measured by latex particle phagocytosis.

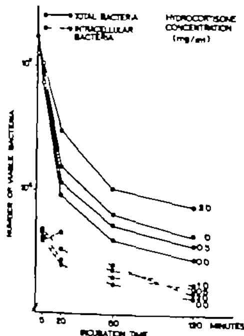


Fig 1 Counts of viable bacteria during incubation of granulocyte-bacteria suspensions with hydrocortisone (mean of 12 experiments) As regards the total number of viable bacteria, the impaired reduction caused by 0.5 1 and 2 mg hydrocortisone per ml is significant ($p < 0.05$) As regards the number of viable intracellular bacteria, the changes caused by hydrocortisone were not significant.

Drug

Complex of 1 mol bis-hydrocortisone (21)-sodiophosphate and 1 mol hydrocortisone (Actocortine Frederiksberg Chemiske Fabrikker A/S, Copenhagen, Denmark) dissolved in distilled water (100 mg per ml) Hydrocortisone, which is loosely bound to phosphate, is split off immediately in the presence of serum

Phagocytic and Bactericidal Activity

The test procedure has been described in detail previously (17 18) To 0.1 ml leukocyte suspension, containing 5×10^{6} neutrophils, 0.4 ml 25 per cent pooled human serum diluted in HBSS and 0.1 ml suspension of *Staphylococcus aureus* "Oxford Heatly strain ($15-20 \times 10$ colony-forming units per ml) was added 0.4 ml hydrocortisone diluted in HBSS. This provided 3–4 bacteria per neutrophil granulocyte, final concentration of 10 per cent serum and a final concentration of hydrocortisone as indicated in the figures.

The tubes were incubated at 37 C and samples were removed at prescribed interval for determination of the total number of viable bacteria and the number of viable intracellular bacteria. The bactericidal capacity of the granulocytes is proportional to the total number of bacteria killed and inversely proportional to the total number of viable bacteria or the number of viable intracellular bacteria. The number of bacteria phagocytized equals the number of viable intracellular bacteria plus the number of bacteria killed.

Controls consisted of bacteria in HBSS containing 10 per cent serum mixed with hydrocortisone in order to detect whether the drug had any direct influence on the bacteria.

Measurement of $^{14}CO_2$ and Lactate Production

Leukocytes ($2.1-7.1 \times 10^{6}$) were incubated for 1 hour in 1 ml Krebs-Ringer bicarbonate buffer containing 0.5 mg glucose and 0.5 μCi (U-^{14}C)glucose and in phagocytosis experiments latex particles. Further details of the incubation procedure and the measurement of $^{14}CO_2$ have been described previously (8) The lactate production was measured by the method of *Barker & Summerson* (3)

Statistical Methods

The results were evaluated by means of the Wilcoxon test of paired differences.

RESULTS

Phagocytosis and Intracellular Killing of Bacteria

In the tests without hydrocortisone, a marked reduction in the total number of viable bacteria was observed and very few bacteria remained viable after incubation for 2 hours (Fig 1) thus demonstrating rapid phagocytosis and intracellular killing of bacteria. In the tests with 0.5 to 2 mg hydrocortisone, significantly larger numbers of bacteria remained viable after incubation for 2 hours, and the numbers of viable bacteria located intracellularly showed only minor and insignificant deviations from the control values (Fig. 1) thus demonstrating significantly impaired phagocytosis but normal or nearly normal intracellular killing of bacteria. Hydrocortisone in concentrations less than 0.5 mg per ml did not influence the granulocyte function. The steroid had no direct effect on the bacteria.

Acta path. microbiol. scand Sect. C 85 284–288 1977

INFLUENCE OF HYDROCORTISONE ON GRANULOCYTE FUNCTION AND GLUCOSE METABOLISM

KAREN ELINA CHRISTIE, BERIT KJOSEN and CLAUS O SOLBERG

Medical Dept. B, University of Bergen, Bergen, Norway

Christie K. E., Kjosen, B. & Solberg, C. O Influence of hydrocortisone on granulocyte function and glucose metabolism. Acta path microbiol. scand Sect. C, 85 284–288 1977

Examination has been made of the influence of hydrocortisone on the *in vitro* phagocytosis and intracellular killing of *Staphylococcus aureus* by human neutrophils and the production of lactate and CO_2 during phagocytosis of latex particles. In high concentrations, 0.5^{-3} mg per ml hydrocortisone caused a significant reduction in the phagocytosis and the production of lactate. Neither the bactericidal activity nor the production of $^{14}CO_2$ from (U-^{14}C)glucose in phagocytizing leukocytes was influenced by these hydrocortisone concentrations.

Key words glucose metabolism granulocytes hydrocortisone intracellular killing phagocytosis.

C. O Solberg Medical Department B, N 5016 Haukeland sykehus, Norway

Received 15.ii.77 Accepted 18.iii.77

Evidence is now accumulating that commonly used antiinflammatory drugs may influence granulocyte function (13) In high concentrations, glucocorticosteroids inhibit some of the biochemical events triggered in granulocytes by ingestion of bacteria (6 10 11) However the importance of these observations for the phagocytosis and intracellular killing of bacteria has not been definitely settled (1 7 14 22) and the results are sometimes conflicting (11 14) This may be due in part to difficulties in methodology particularly in the separation of extracellular and intracellular bacteria in an *in vitro* phagocytic system.

In the present study the influence of hydrocortisone on the phagocytic and bactericidal activities of human neutrophils has been examined, using a recently developed method which facilitates a precise *in vitro* evaluation of each function (17 18) As the phagocytic and bactericidal activities are dependent on certain metabolic processes in the leukocytes, the influence of hydrocortisone on the leukocyte glucose metabolism, as expressed by the production of CO_2 and lactate, has been examined and compared with the results of the granulocyte-bacteria experiments.

MATERIAL AND METHODS

Leukocytes

Leukocytes were obtained from healthy human donors by Isopaque/dextran sedimentation of heparinized blood, as described previously (17) The cells were suspended in Hanks balanced salt solution (HBSS) containing 1 per cent gelatin Eighty-four to 90 per cent of the leukocytes were neutrophil granulocytes, and 96 to 99 per cent of the neutrophils resisted staining with trypan blue and retained their functional integrity intact as measured by latex particle phagocytosis.

activity of the granulocytes was significantly ($p < 0.05$) reduced by 0.5–2 mg hydrocortisone per ml. At those concentrations the drug caused a slight, but not significant, reduction in the bactericidal activity of the granulocytes, thus indicating that the influence of hydrocortisone on the leukocyte function takes place mainly in the engulfment phase and that the influence on the bactericidal activity of the leukocytes is minimal. This is also supported by our results of leukocyte glucose metabolism.

Most of the CO_2 produced by the granulocytes is derived from the pentose phosphate pathway which reflects the processes activated to kill ingested bacteria (9). The influence of hydrocortisone on the production of $^{14}CO_2$ from (U-^{14}C)glucose indicates no inhibiting effect of the steroid on the pentose phosphate pathway. As verified by measurement of viable intracellular bacteria, this suggests that hydrocortisone has little influence on the bactericidal activity of the granulocytes.

The leukocytes seem to derive most of the energy required for the ingestion process from anaerobic glycolysis (12). As the leukocytes oxidize very little glucose through the citric acid cycle, lactate can be considered as the end product of glycolysis (21). The production of lactate in the leukocytes was reduced by the same concentrations of hydrocortisone as those which reduced phagocytosis. It has been suggested that hydrocortisone may alter the leukocyte IgG and complement receptors (15, 16) and consequently inhibit the binding of opsonized bacteria (5, 19). The inhibition of lactate production observed in nonphagocytizing leukocytes in the absence of serum indicates, however, that hydrocortisone also inhibits the glycolytic activity independent of IgG, complement and other serum factors, and independent of phagocytosis. The consequence of the reduction in glycolytic activity will be less production of energy. The lack of energy available for the ingestion of bacteria or latex particles seems a reasonable explanation of the reduced phagocytosis observed with high concentrations of hydrocortisone in the medium.

Our results are in agreement with the findings of others of significantly reduced engulfment of bacteria (2) and latex particles (6, 10) by human leukocytes in the presence of high concentrations of hydrocortisone. Other studies suggesting that corticosteroids may inhibit the intracellular killing of bacteria (1, 4, 20) are not supported by our results.

In the granulocyte-bacteria experiments, less than 0.5 mg hydrocortisone per ml caused only minor and insignificant effects on the phagocytic and bactericidal activities of the granulocytes. However, the measurement of $^{14}CO_2$ and lactate production indicates that lower concentrations of hydrocortisone also have an effect, and that that effect may be different from what is observed with higher concentrations.

The study was supported by the *Norwegian Cancer Society*.

REFERENCES

1. *Baardsen, A., Trippestad, A. & Midtvedt, T.*: In vitro influence of glucocorticosteroids on opsonic and bactericidal activities against *Escherichia coli* in rat serum. Acta path. microbiol. scand. Sect. B *82*, 696–700, 1974.
2. *Baardsen, A.*: Influence of hydrocortisone on uptake and elimination of ^{32}P-labelled E. coli by rat polymorphonuclear neutrophils (PMN) in vitro. Acta path. microbiol. scand. Sect. C, *84*, 131–139, 1976.
3. *Barker, S. B. & Summerson, W. H.*: The colorimetric determination of lactic acid in biological material. J. Biol. Chem. *138*, 535–554, 1941.
4. *Chretien, J. H. & Garagusi, V. F.*: Corticosteroid effect on phagocytosis and NBT reduction by human polymorphonuclear neutrophils. J. Reticuloendothel. Soc. *11*, 358–367, 1972.
5. *Christie, K. E., Solberg, C. O., Larsen, B., Grewe, A. & Tønder, O.*: Influence of IgG (F(ab')₂) and IgM on the phagocytic and bactericidal activities of human neutrophil granulocytes. Acta path. microbiol. scand. Sect. C, *84*, 119–123, 1976.
6. *Cooper, M. R., DeChatelet, L. R. & McCall, C. E.*: The in vitro effect of steroids on polymorphonuclear leukocyte metabolism. Proc. Soc. Exp. Biol. Med. *141*, 986–990, 1972.

Fig 2 Effect of hydrocortisone on $^{14}CO_2$ production from (U ^{14}C)glucose by 10^7 leukocytes during 1 hour of incubation. The mean values ± S.D for 12 experiments (in duplicate) are recorded. As regards phagocytizing leukocytes, the decrease caused by 0 03 and 0 12 mg was significant ($p < 0 02$) As regards nonphagocytizing leukocytes, the increase was significant for all values of hydrocortisone ($p \leq 0 01$)

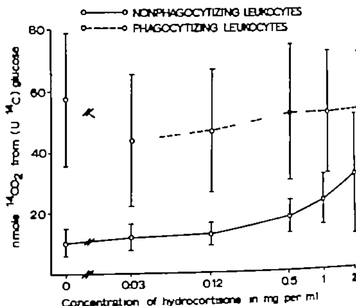
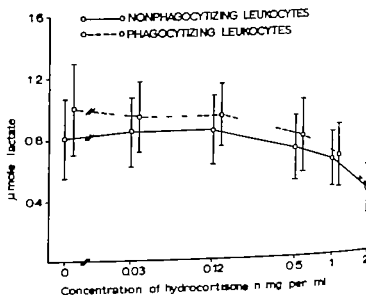


Fig 3 Effect of hydrocortisone on the lactate production by 10^7 leukocytes during 1 hour of incubation. The mean values ± S.D for 12 experiments (in duplicate) are recorded. As regards phagocytizing leukocytes, the decrease caused by 0 5 1 and 2 mg hydrocortisone per ml was significant ($p < 0 01$) As regards nonphagocytizing leukocytes, the increase caused by 0 03 and 0 12 mg and the decrease caused by 1 and 2 mg were significant ($p < 0 01$)



Leukocyte Glucose Metabolism

In nonphagocytizing leukocytes, the production of $^{14}CO_2$ from (U ^{14}C)glucose increased with increasing concentration of hydrocortisone (Fig 2) In leukocytes phagocytizing latex particles, the influence of hydrocortisone on the $^{14}CO_2$ production was more complex, viz. hydrocortisone concentrations below 0.5 mg per ml produced slight inhibition while concentrations above that level had no significant effect. The production of lactate in both nonphagocytizing and phagocytizing leukocytes was reduced markedly by hydrocortisone in concentrations of 0.5 to 2 mg per ml (Fig 3) thus indicating that the steroid inhibited the production of energy available for the phagocytic process. In concentrations below 0 5 mg per ml, hydrocortisone had a slightly stimulating effect on the lactate production in nonphagocytizing leukocytes but had no influence on the production in phagocytizing leukocytes.

DISCUSSION

The precise mechanisms for the action of glucocorticoids on phagocytic cells remain unknown. In the present study the phagocytic

Acta path. microbiol. scand. Sect. C, 85: 289–296, 1977

NEUTROPHIL GRANULOCYTE CHEMOTAXIS *IN VITRO*

Comparison of the Response to Casein and a Bacterial Chemotactic Factor and Evaluation of an Automatic Method for Counting Cells on a Membrane Filter Surface

N. H. VALERIUS

Statens Seruminstitut, Department of Clinical Microbiology, Rigshospitalet, and Department of Infectious Diseases M, Rigshospitalet, Copenhagen, Denmark

Valerius, N. H. Neutrophil granulocyte chemotaxis in vitro. Comparison of the response to casein and a bacterial chemotactic factor and evaluation of an automatic method for counting cells on a membrane filter surface. Acta path. microbiol. scand. Sect. C, 85: 289–296, 1977.

A method is described for evaluating neutrophil granulocyte chemotaxis *in vitro* using a modified reversible Boyden chamber. The variation in cell detachment from the attractant surface of the filter after the migration through the filter was shown to be eliminated by reversing the chambers during the incubation period. In unreversed chambers, the loss of cells from the bottom surface of the filter was much higher on attraction with casein than with a bacterial chemotactic factor (BCF). The finding of different kinetic response to casein and to BCF may suggest differing affinity to various chemoattractants within circulating neutrophils. The results obtained after introducing an automatic quantitative image analysis system for the counting of the cells at the filter surface are found to be very well correlated to those with conventional direct microscope counting.

Key words: Chemotaxis; neutrophil granulocyte; casein; bacterial chemotactic factor; *in vitro*.

N. H. Valerius, Department of Clinical Microbiology 7806, Rigshospitalet, Tagensvej 18, DK 2200 Copenhagen N, Denmark.

Received 11.ii.77. Accepted 15.iii.77.

The introduction by *Boyden* in 1962 (2) of an *in vitro* assay for the measurement of leukocyte chemotaxis using a micropore filter technique has provided a wealth of information regarding substances which possess chemotactic activity and factors which influence the cellular response. In the classical Boyden technique, the chemotactic activity is measured by counting the number of cells adhering to the bottom surface of the membrane filter through which they have migrated. However, recent reports (6, 13, 14) have stressed the inaccuracy of this method, since an unpredictable and variable number of cells detach from the filter after their arrival at its bottom surface, thus eluding enumeration. Several modifications of the Boyden technique for the elimination of this source of error have been reported. In the front-line technique, the application of a short incubation time does not permit the cells to reach the bottom surface, so the chemotactic activity can be expressed by the distance mi-

7 *Dale D C* & *Petersdorf R G* Corticosteroids and infectious diseases. Med Clin. North. Am. *57* 1277–1287 1973

8 *Kjosen B Bassoe H H* & *Myking O* The glucose oxidation in isolated leukocytes from female patients suffering from overweight or anorexia nervosa. Scand. J clin. Lab. Invest. *35* 447–454 1975

9 *Klebanoff S J* Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes. Seminars in Hematology *12* 117–142 1975

10 *Kvarstein B* & *Stormorken H.*. Influence of acetylsalicylic acid, butazolidine, colchicine, hydrocortisone chlorpromazine and imipramine on the phagocytosis of polystyrene latex particles by human leucocytes. Biochem. Pharmac. *20* 119–124 1971

11 *Mandell G L. Rubin W* & *Hook E. W* The effect of an NADH oxidase inhibitor (hydrocortisone) on polymorphonuclear leukocyte bactericidal activity J Clin. Invest. *49* 1381–1388 1970

12 *Quie P G* Disorders of phagocyte function. Year Book Medical Publishers, Inc. Chicago, 1972

13 *Quie P G* Pathology and bactericidal power of neutrophils. Seminars in Hematology *12* 143–160 1975

14 *Ruutu T* & *Konsunen T U* *In vitro* effect of antiinflammatory agents on phagocytosis and bactericidal killing by human neutrophilic leucocytes. Acta pharmacol. et toxicol *31* 226–237 1972.

15 *Schreiber A D Parrons J McDermott, P* & *Cooper R. A.*. Effect of corticosteroids on the human monocyte IgG and complement receptors. J Clin. Invest. *56* 1189–1197 1975.

16 *Sneiderman C A* & *Wilson J W* Effects of corticosteroids on complement and the neutrophilic polymorphonuclear leucocyte. Transplant. Proc. *7* 41–48, 1975

17 *Solberg C O* Enhanced susceptibility to infection. A new method for the evaluation of neutrophil granulocyte functions. Acta path microbiol. scand. Sect. B, *80* 10–18, 1972.

18. *Solberg C O* Protection of phagocytized bacteria against antibiotics. A new method for the evaluation of neutrophil granulocyte function. Acta med. scand. *191* 383–387 1972.

19 *Solberg C O., Christie K. E., Larsen B* & *Tonder O* Influence of antibodies and thermolabile serum factors on the bactericidal activity of human neutrophil granulocytes. Acta path. microbiol, scand. Sect. C, *84* 112 118, 1976

20 *Spirer Z. Zakuth I Weisman Y* & *Bogair A* The effect of corticosteroids on the bactericidal activity of leococytes. Experientia *30* 303–304 1974

21 *Stjernholm R. L. Burns C P* & *Hohnadel, J H* Carbohydrate metabolism by leukocytes. Enzyme *13* 7–31 1972.

22 *Zurier R. B* & *Weissmann G* Anti-immunologic and anti inflammatory effects of steroid therapy Med Clin. North. Am. *57* 1295–1307 1973

dept with malinol (Chroma, Stuttgart, Germany) with the attractant side upwards.

Counting: Counting by direct microscopy was performed at 25 × 10 amplification, and the chemotactic activity was expressed as the mean number of cells within an ocular grid 1 × 1 cm which had completely traversed the filter lying on its attractant surface. In each filter five fields were counted, four of which were equidistant to the cell edge on the filter and the fifth as near to the centre of the filter as possible. Automatic counting was performed with a Classimat (Leitz, Wetzlar, Germany) at 10 × 10 amplification along two diameters perpendicular to each other in the filter. The television screen field covered 0.2145 mm² and between 25 and 30 fields per filter were counted, constituting 11–13 per cent of the cell-coated filter surface. Chemotaxis was expressed as the mean number of cells per television screen field. All samples were run in duplicate or triplicate, and the results represent the mean or median. In all experiments a simultaneous assessment of the spontaneous migration was included by incubating the cells without a chemoattractant.

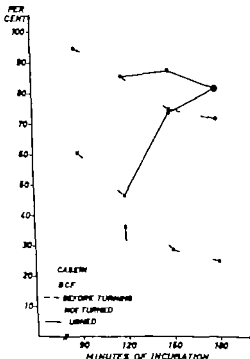


Fig. 1 A. Number of cells adhering to the attractant surface of the filter as percentage of the total number of cells migrating through the filter on attraction with casein and BCF at varying incubation times. The chambers were either turned upside down (——) or left without being reversed (– – –) after 120 minutes incubation (arrow). Automatic counting. Mean of two experiments.

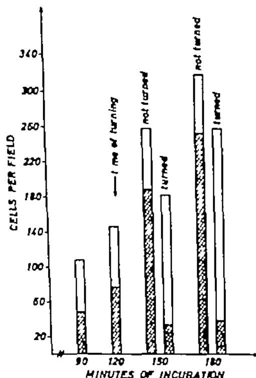


Fig. 1 B. Distribution and absolute number of cells per field on the attractant surface of the gradient filter and on the bottom filter on attraction with casein in reversed and unreversed chambers at varying incubation times. The bars represent the total number of cells migrating through the filter. The hatched areas denote the number of cells on the bottom filter and the open areas the number of cells on the attractant surface of the gradient filter. One of the two experiments from Fig. 1 A.

RESULTS

Effect of reversing the chambers. Fig. 1 A shows the number of cells adhering to the attractant surface of the filter as percentage of the total number of cells which had migrated through the filter. This was determined as the sum of the number of cells adhering to the attractant surface of the gradient filter and the number of cells lying on

grated into the filter by the furthest moving cells In the double-filter technique (6), a cell impermeable filter is placed immediately below the conventional filter and the chemotactic activity is measured by counting the number of cells at the interface between the two filters. This involves, however the counting of two filters for each sample thus rendering it unsuitable in practice. Another double filter technique uses *radioactive labelling* of the leukocytes and subsequent recording of the incorporation of radioactivity into the lower filter (4 5) In 1975 *Jungi* (7) introduced the reversible Boyden chamber During the incubation period this chamber can be turned upside down thus preventing the cells from falling off the filter after their arrival at its attractant surface. The present report analyses this method further using *two different* chemoattractants, and introduces an improved counting method using an automatic quantitative image analysis system.

MATERIALS AND METHODS

Leukocytes Venous blood was drawn into a plastic syringe containing heparin without preservative (Novo, Copenhagen) for a final concentration at 10 Iu heparin per ml blood Half-volume 5 per cent Dextran (molecular weight 250 000 in 0.154 M saline) was mixed with the blood, which was left for 45–60 minutes at room temperature for sedimentation of the erythrocytes. The buffy coat was withdrawn in sterile disposable polystyrene tubes (1202 Nunc, Roskilde, Denmark) After centrifugation at 250 × *G* for 10 minutes, the supernatant was aspirated and contaminating erythrocytes in the cell pellet were lysed twice by suspending the cells in cold saline 0 2 per cent for 30 seconds, followed by the addition of an equal volume of saline 1 6 per cent. The cells were finally suspended in Gey's medium (containing 2 per cent human albumin (Statens Seruminstitut) 167 I u. penicillin (Novo Copenhagen) per ml and 50 μg per ml streptomycin (Rosco, Copenhagen) adjusted to pH 7 2) at a final concentration of 1×10^6 polymorphonuclear granulocytes (PMN) per ml.

Chemotactic factors A stock solution of casein (nach Hammarsten, Merck, Darmstadt, Germany) 5 per cent in 0 154 M saline adjusted to pH 7 0 with NaH_2PO_4 was autoclaved and kept in 3 ml aliquots at −20 C until use. After thawing, the pH was adjusted to 7.2 with NaOH 0 1 N and the solution was diluted 1 10 with Gey's medium containing antibiotics, as described. For the preparation of a bacterial chemotactic factor (BCF) *Escherichia coli* type 0?:K1 H4 (kindly provided by Dr. *Ida Orskov* Statens Seruminstitut) was grown for 24 hours at 35 C in TC medium 199 (Difco, Detroit, Michigan) After centrifugation at 10,000 × *G* for 30 minutes, the supernatant was passed through a 0.22 micron millipore filter (Millex, Millipore Bedford, Mass.) and kept in 4 ml aliquots at −20 C until use pH was adjusted to 7.2 with 0 1 N NaOH before use, and the filtrate was diluted 1.3 with Gey's medium, as described.

Chemotaxis chambers These were made of acryl plast (Molytex, Rodovre, Denmark) from a model exactly as described by *Jungi* (7) They consisted of three parts (1) a lower part with the attractant compartment, on the top of which the filter was placed and which could be filled through a lateral hole (2) a cylindrical part provided with a thread to ensure tight fitting with the lower part, thus fixing the filter and serving as the cell compartment and (3) a cover with a small central hole allowing air escape. Each compartment contained 0.5 ml.

Filters Cellulose nitrate membrane filters with a pore size of 3 microns (11302 Sartorius, Göttingen, Germany) were used for all experiments with PMN For experiments in which a cell-impermeable bottom filter was placed in the chamber this had a pore size of 0.65 microns (DAWP Millipore Bedford Mass.) Filters with a diameter of 13 mm were punched out from a large sheet for reasons of economy Filters from one single sheet were used on each day to ensure uniformity

Handling of the chambers After fixing the filter between the two compartments, the lower (attractant) compartment was filled through a hypodermic needle inserted in the lateral hole When the filter had become soaked, the needle was removed and the hole was sealed with waterproof adhesive tape. Immediately afterwards, 0.5 ml cell suspension was pipetted into the upper compartment which was closed with the cover and the hole sealed with another strip of adhesive tape. Incubation was performed at 37 C for 180 minutes. After 120 minutes incubation, the chambers were cautiously turned upside down. After incubation, the cover tape and the cover were removed and the fluid in the cell compartment was aspirated. After separating the two compartments the filter was removed and immediately put into 96 per cent ethanol for fixation of the cells. After 12 minutes the filters were stained with haematoxylin Meyer rinsed in distilled water and dehydrated in ethanol before clearing in xylene They were finally mounted on slides under cover

dps with malinol (Chroma, Stuttgart, Germany) ith the attractant side upwards.

Counting: Counting by direct macroscopy was performed at 25 × 10 amplification, and the chemotactic activity was expressed as the mean number of cells within an ocular grid 1 × 1 cm which had completely traversed the filter lying on its attractant surface. In each filter five fields were counted, four of which were equidistant to the cell edge on the filter and the fifth as near to the centre of the filter as possible. Automatic counting was performed with a Classimat (Leitz, Wetzlar Germany) at 10 × 10 amplification along two diameters perpendicular to each other in the filter. The television screen field covered 0.2145 mm² and between 25 and 30 fields per filter were counted, constituting 11–15 per cent of the cell-coated filter surface. Chemotaxis was expressed as the mean number of cells per television screen field. All samples were run in duplicate

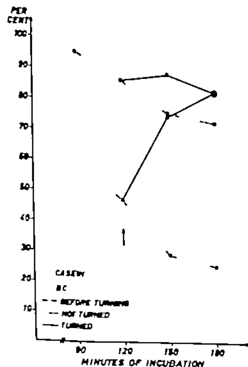


Fig. 1 A. Number of cells adhering to the attractant surface of the filter as percentage of the total number of cells migrating through the filter on attraction with casein and BCF at varying incubation times. The chambers were either turned upside down (——) or left without being reversed (– – –) after 120 minutes incubation (arrow). Automatic counting. Mean of two experiments.

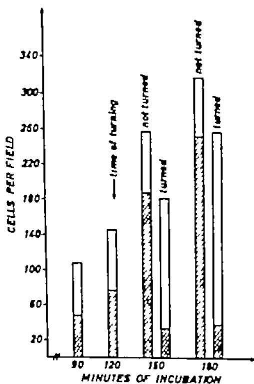


Fig. 1 B. Distribution and absolute number of cells per field on the attractant surface of the gradient filter and on the bottom filter on attraction with casein in reversed and unreversed chambers at varying incubation times. The bars represent the total number of cells migrating through the filter. The hatched areas denote the number of cells on the bottom filter and the open areas the number of cells on the attractant surface of the gradient filter. One of the two experiments from Fig. 1 A.

or triplicate, and the results represent the mean or median. In all experiments a simultaneous assessment of the spontaneous migration was included by incubating the cells without a chemoattractant.

RESULTS

Effect of reversing the chambers. Fig. 1 A shows the number of cells adhering to the attractant surface of the filter as percentage of the total number of cells which had migrated through the filter. This was determined as the sum of the number of cells adhering to the attractant surface of the gradient filter and the number of cells lying on

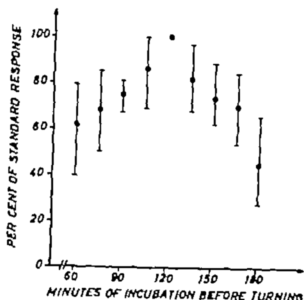


Fig 2 Effect of varying the time for reversing the chambers during an incubation period of 180 minutes on the number of cells adhering to the attractant surface of the filter on attraction with casein. The results are expressed as percentage of the standard response (chambers reversed after 120 minutes incubation) Direct counting Mean and range of three experiments.

number of cells on the attractant surface of the gradient filter occurred between 90 and 180 minutes in the unreversed chambers. This reflects a state of equilibrium between cell detachment and arrival of new cells at this surface, since the cell number on the bottom filters continues to increase. It also appears that after their detachment and subsequent collection on the bottom filter cells may return to the gradient filter on reversing the chambers, since the bottom filters contain a larger number of cells before reversing than after reversing the chambers. The total number of migrating cells is greater in unreversed than in reversed chambers. This was also seen in BCF stimulated samples (not shown here)

Effect of varying the reversion time of the chambers Fig 2 shows the variation in the number of cells adhering to the attractant surface of the filter on reversing the cham-

an additional cell impermeable filter placed in the bottom of the attractant compartment of each chamber Chambers were incubated for different periods of time and those incubated for longer than 120 minutes were either reversed then or left without being reversed Stimulation was performed with either casein or BCF After 90 and 120 minutes incubation i e. before reversing the chambers, a much lower proportion of the total number of migrating cells was found adhering to the attractant surface of the gradient filter in casein-stimulated samples than in BCF stimulated samples This difference was increased in the chambers that were left unreversed at 120 minutes. However 30 minutes and more after reversing the chambers, the proportion was found to be equal in casein stimulated and BCF-stimulated samples and much higher than in the unreversed casein stimulated samples. It can be seen from Fig 1 B, which shows the distribution of cells on the two filters in absolute numbers at various times during stimulation with casein that no increase in the

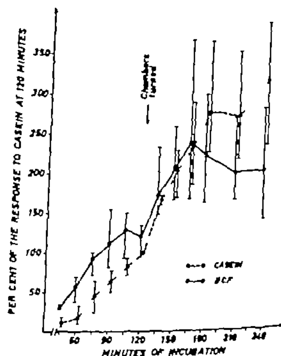


Fig 3 Time-course of neutrophil granulocyte chemotaxis to casein and BCF The results are expressed as the number of cells on the attractant surface of the filter as percentage of the number at 120 minutes using casein as attractant. Automatic counting Mean and range of three experiments.

TABLE 1. *Number of PMN on the Attractant Surface of the Filter in Varying Concentration Gradient and at Varying Absolute Concentrations of Casein. Incubation Time 180 Minutes Mean of Two Experiments*

		Concentration of casein (mg/ml) in the attractant compartment			
		0	1	2.5	5
Concentration	0	0	52	170	231
of casein (mg/ml)	1	6	57	106	181
in the cell	2.5	7	40	98	120
compartment	5	15	28	53	112

bers at different times during an incubation period of 180 minutes. The maximum number of cells was found when the chambers were reversed at 120 minutes.

Kinetics of the chemotactic activity to casein and BCF Fig. 3 shows the kinetics of neutrophil chemotactic response to casein and to BCF For comparison purposes, the results are expressed as percentage of the response at 120 minutes to casein in each experiment. The chemotactic activity to casein was found to increase throughout an incubation period of 240 minutes, with a maximum increase between 90 and 180 minutes, while a peak activity to BCF occurred at 150–165 minutes. The migration on stimulation with BCF was much stronger than with casein during the first 90 minutes of incubation. On reversing the chambers at 120 minutes, the activity became equal until the peak activity to BCF was reached, resulting in a higher final chemotactic activity to casein than to BCF

Effect of varying the concentration gradient and the absolute concentration of casein. The influence of the direction of the concentration gradient and the absolute concentration of casein on the number of cells migrating through the filter is shown in Table 1 In these experiments the cells were allowed to migrate through the filter in positive, i.e. from lower towards higher concentrations of casein, and negative concentration gradients and without gradients at different concentrations of casein. Along the diagonal from upper left to lower right in the table, the absolute concentration of casein increases in the absence of a concentration gradient across the filter The activity increased with increasing concentrations of casein. The concentration gradient was positive above this diagonal and negative below it. The generally higher figures above the diagonal than below it indicate a uni-directional locomotion of granulocytes towards casein. The influence of the concentration gradient at various absolute concentrations of casein is seen along the parallel lines from lower left to upper right in the table. The concentration gradients reversed from being negative to being positive along these lines, while the absolute concentration of casein remained almost constant on each line. The cell number is seen to increase along all these lines. Without chemoattractant no cells migrated through the filter in these experiments. In numerous other control studies a maximum of 5–10 cells per field was usually observed

Influence of the concentration of PMN Fig. 4 shows the effect of varying the PMN concentration on the number of cells traversing the filter when attracted by casein. In a semi-logarithmic scale this relation appears to be linear

Comparison of direct counting and automatic counting Fig 5 gives the relationship between the results from counting by direct microscopy and automatic counting 140 filters from four different days, in which the cell count ranged from 0–274 per field by variations in the casein concentration, were counted by both methods. Since the points in the figure assumed a fan-shaped distribu-

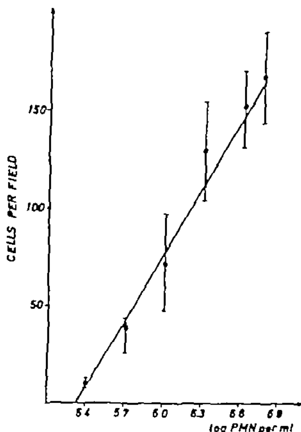


Fig. 4. Chemotactic activity at various neutrophil granulocyte concentrations in the cell compartment. Results were obtained by the standard procedure: total incubation time of 180 minutes and reversing of the chambers after 120 minutes incubation. Direct counting. Mean and range of three experiments.

tion the calculations were performed on logarithmic values to the cell counts (r = 0.98). Higher values were obtained by automatic counting than by direct counting. The proportion between the two values was found to be conversely related to the absolute cell number.

Reproducibility of the method. The standard deviation was found repeatedly to be less than 11 per cent of the mean when running from 6–24 simultaneous identical tests.

DISCUSSION

The classical Boyden chamber for assaying leukocyte chemotaxis *in vitro* has been modified by the construction of a chamber that can be reversed during the incubation period, in order to prevent loss of cells from the attractant surface of the micropore filter (7). The importance of eliminating this source of error for a quantitative analysis of the chemotactic activity can be seen from Fig. 1 which shows that the cell loss is much higher on attraction with casein than with BCF. It has been suggested previously (6, 13) though not reported that the degree of cell detachment from the filter during the incubation period is influenced by the chemotactic factor used. Whether this difference between casein and BCF represents an effect on the filter matrix rendering it more or less sticky or is the result of a different adhesiveness of the PMN on exposure to different chemotactic factors, is under investigation at present. The difference observed in cell detachment may help to explain the highly conflicting results of

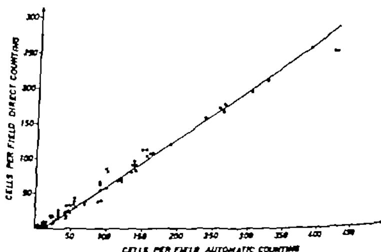


Fig. 5. Relationship between the results by direct and automatic counting of 134 filters (r = 0.98) calculated from logarithmic values.

investigations of PMN chemotaxis in various disease states reported from different groups (1, 3, 8, 9 10 11) The finding of a larger total number of migrating cells in unreversed chambers than in reversed chambers (Fig 1B) supports a previous report (7) in which this was ascribed to an effect of gravity Fig 2 shows that the highest chemotactic activity to casein occurred when the chambers were reversed at 120 minutes during a 180 minute incubation period. This time for reversing the chambers is considered optimal, since the adverse effects of cell migration against gravity and cell detachment from the filter seem to be minimized then. The kinetic study of the response of the PMN to attraction with casein and BCF reveals different patterns (Fig. 3) Initially the response to BCF is far stronger than to casein, but it levels off sooner and reaches a peak at 150–165 minutes incubation. The maximal increase in the response to casein was seen between 90 and 180 minutes incubation. The different loss of cells during the first 120 minutes incubation i.e. before reversing the chambers, is not sufficient to explain fully this different kinetic behaviour since more than half of the cells are retained on the gradient filter in casein-stimulated samples during this period. Also experiments in which the total number of migrating cells was determined show this kinetic difference. The observation may suggest that BCF possesses a strong attractant effect on a relatively small proportion of the PMN while casein exerts a weaker effect on a larger proportion of the cells

It has been pointed out by *Zigmond & Hirsch* (14) and *Wilkinson* (15) that granulocytes show enhanced random migration in the mere presence of a chemotactic agent. An increased leukotactic response observed in a micropore filter system might thus be a reflection of this rather than chemotaxis, i.e. directed locomotion along a concentration gradient Since previous studies for analysis of the contribution to the activity of the two types of stimulation observed have been performed with the front line technique (14 15) it was considered pertinent to investigate whether the distinction between stimulated random and directed locomotion would be possible by the present method. The results shown in Table 1 are in close agreement with those of *Zigmond & Hirsch* (14) and *Wilkinson* (15) The number of cells at the attractant surface of the filter thus represents the combined effect of stimulated random migration and chemotaxis.

The observation of a log-linear relationship between the number of granulocytes deposited on the filter and the number of cells at the attractant surface supports previous findings of a linear relationship within only a narrow range of the number of cells (12) The observation re-emphasizes the importance of a strict control of the concentration of the PMN in the cell suspension.

Counting by conventional microscopy of the cells on the attractant surface of the filter is extremely tedious and time-consuming, and represents the limitation in practice of the number of samples that can be examined per day The application of an automatic quantitative image analysis system eliminates this disadvantage since each filter can be evaluated in less than two minutes. The figures obtained by automatic counting are higher than those obtained by direct counting, in spite of a smaller field of vision. This is due to the registration also of some of the cells lying immediately below the surface and to a far less extent to the registration of occasional impurities on the filter The amount of impurities is independent of the chemotactic activity and proportionately more cells will perform a full penetration of the filter on strong stimulation. This explains why the mean proportion between the results of automatic and direct counting decreases with increasing absolute number of cells. However the good correlation observed between the results of direct and automatic counting seems to show that these sources of error are of minor importance. Since automatic counting permits evaluation of a far larger area of the filter than is possible in practice by direct counting, it would seem justifiable to assume that the results of the

automatic counting give a better description of the chemotactic activity in the occasional filter in which an uneven distribution of the cells is observed.

The method described offers a number of advantages. It presents an effective elimination of the serious source of error in the detachment of the cells from the filter It has good reproducibility and has been shown (7) to be suitable for measuring monocyte and eosinophil chemotaxis also thus facilitating comparative studies. It does not involve radioactive labelling of the cells. Handling of the chambers is easy and quick and permits the handling of more than 100 samples a day The introduction of an automatic image analysis system is extremely expensive, but once having obtained one rapid highly reliable evaluation of the filters is achieved

---

This study was supported by the *Danish Medical Research Council*. Mrs. *Annette Winkel* and Mrs. *Hanne Tamstorf* are thanked for excellent technical assistance. Thanks are due to Mr *M Weiss Bentzon* for performing the statistical analysis, and to professor *V Faber* and Dr *Chr Koch* for critical review of the manuscript.

REFERENCES

1 *Baisero M H* Chimiotactisme des polynucleaires humains in vitro III Etude de l'infection aiguë et chronique de l'adulte. Schweiz. Med. Wschr *103* 1599–1603 1973

2 *Boyden S* The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. J Exper Med. *115* 453–466 1962.

3 *Clark R A Kimball H R & Decker J L.* Neutrophil chemotaxis in systemic lupus erythematosus. Ann. Rheum. Dis. *33* 167–172, 1974

4 *Gallin J I Clark R. A & Kimball, H R.* Granulocyte chemotaxis an improved in vitro assay employing ^{51}Cr-labeled granulocytes. J Immunol. *110* 233–240, 1973.

5. *Goetzl E. J & Austen K F.* A method for assessing the in vitro chemotactic response of neutrophils utilizing ^{51}Cr labeled leukocytes Immunol. Commun. *1* 421–430 1972.

6 *Keller H U., Borel J F., Wilkinson P C, Hess M W & Cottier H* Re-assessment of Boyden's technique for measuring chemotaxis J Immunol. Meth. *1* 165–168, 1972.

7 *Jungi T W* Assay of chemotaxis by a reversible Boyden chamber eliminating cell detachment. Internat. Arch. All. Appl. Immunol. *48* 341–352, 1975

8. *Leimgruber A., Frei P C., Ochsner M Schubert M & Senden T* Chimiotactisme des polynucleaires humains in vitro. Etude dans les maladies rheumatismales inflammatoires. Schweiz. Med. Wschr *105* 1730–1732 1975

9 *McCall C E. Caves J Cooper R & deChatelet L.* Functional characteristics of human toxic neutrophils. J Infect. Dis. *124* 68–75 1971

10 *Mowat A & Baum J.* Chemotaxis of polymorphonuclear leucocytes from patients with rheumatoid arthritis. J Clin. Invest. *50* 2541–2547 1971

11 *Mowat A & Baum J* Polymorphonuclear leucocyte chemotaxis in patients with bacterial infections. Brit. Med. J iii, 617–619 1971

12 *Patten E., Gallin J I., Clark R. A & Kimball H R.* Effects of cell concentration and various anticoagulants on neutrophil migration. Blood *41* 711–719 1973

13 *Wilkinson P C* Chemotaxis and inflammation. Churchill Livingstone Edinburgh and London 1974

14 *Zigmond S H & Hirsch J G* Leucocyte locomotion and chemotaxis. J Exper Med. *137* 387–410 1973

Acta path. microbiol. scand. Sect. C, 85 297–300 1977

PRODUCTION OF MONOSPECIFIC ANTISERA AGAINST TWO VACCINIA VIRUS ANTIGENS

INGWILD GURVIN and GUNNAR HAUKENES

The Gade Institute Department of Microbiology University of Bergen, Bergen, Norway

Gurvin, I. & Haukenes, G. Production of monospecific antisera against two vaccinia virus antigens. Acta path. microbiol. scand. Sect. C, 85 297–300 1977

Vaccinia virus antigen was prepared from infected rabbit lung fibroblast monolayers grown in Eagle's medium supplemented with serum obtained from the rabbit going to be used for immunization. Monospecific rabbit antisera against the L and S antigens were produced by immunization with immunoprecipitates cut out from the agarose after quantitative line immunoelectrophoresis using antigen produced in HeLa cells

Key words Vaccinia antigen; antisera; quantitative immunoelectrophoresis.

G Haukenes, Mikrobiologisk avdeling, MHF-bygget, N 5016 Haukeland sykehus, Norway

Received 8.ii.77 Accepted 17.iii.77

Production of rabbit antisera against the antigenic component of immunoprecipitates has been reported by several investigators. *Hansen et al.* (4) using the double diffusion in gel method, produced antisera against IgM and IgG in this way. They concluded that these immunocomplexes were very potent immunogens. *Shivers & James* (6) raised antibodies against oviducal proteins and reached the same conclusion. The same principle has been applied to immunoprecipitates obtained by quantitative immunoelectrophoresis of *Mycobacterium bovis* antigens by *Harboe et al.* (2) and in a study of herpes simplex virus antigens by *Vestergaard* (7). In the latter study a peak of an immunoprecipitate obtained by crossed immunoelectrophoresis and representing an antigen which was present only in herpes simplex virus type 2, was cut out of the agarose gel. Inoculation of the precipitate into rabbit gave a monovalent antiserum which reacted with type 2 virus only.

We have studied the major vaccinia virus precipitinogens by quantitative immunoelectrophoresis methods (5). The need for potent monovalent antisera prompted us to attempt the above-mentioned immunization principle. In addition, special measures were taken to avoid antibody production against non viral host material.

MATERIALS AND METHODS

Virus Strains

The virus strain used was the Smallpox vaccine strain from Statens Seruminstitut, Copenhagen, obtained from Dr *A Harboe* Oslo.

Vaccinia Virus Antigen (VVA)

Primary rabbit lung fibroblast monolayers were obtained by trypsinization of lung tissue from young animals. The cells were grown in Eagle Minimum Essential Medium (MEM) (Grand Island Biological Company NY USA) supplemented with 10 per cent rabbit serum (Flow Labs Irvine, Scotland). Before infection, the medium was changed to Eagle MEM with 2 per cent serum obtained

from the rabbit going to be used for immunization. The cells were infected at high multiplicity with virus propagated in similar cultures. After 48 h the cells were harvested by means of a rubber policeman, frozen and thawed 3 times, sonicated and then centrifuged at 1500 × g for 10 min to remove some cell debris. The supernatant (VVA rabbit lung fibroblasts) containing 1—5 × 10^7 p.f.u. per ml was used for immunization with whole virus. VVA for immunoelectrophoresis (VVA HeLa) was prepared in a similar manner from infected HeLa cells (Bristol strains calf serum adapted, Flow Labs.) grown in Eagle's MEM supplemented with calf serum (Flow Labs.) VVA HeLa contained 0.5–1 × 10^8 p.f.u. per ml.

Immunization with Whole Virus

Rabbits were inoculated once by 4 intradermal injections of VVA rabbit lung fibroblast and bled after 6 weeks. The antiserum was designated D

Immunization with Immunoprecipitates

The immunoprecipitates were produced by line immunoelectrophoresis from an 0 3 × 18 cm ditch filled with VVA HeLa dissolved in melted agarose The antibody-containing agarose was 4 × 18 cm. After electrophoresis for 18 h the plates were washed twice for 30 min with phosphate buffered saline (pH 7.2) and placed in 3 per cent formaldehyde for 5 min to destroy possible infective virus particles. The immunoprecipitates were then cut out of the gel layer frozen once, crushed mechanically and mixed with an equal volume of Freund's complete adjuvant. One injection was given intramuscularly into the rabbits. The animals were bled at weekly intervals starting at the 4th week. Optimal antibody response was obtained after 5 to 7 weeks.

Quantitative Immunoelectrophoresis

Line and crossed line immunoelectrophoresis was performed as described in (1 3) The antisera were mixed with agarose to a final dilution of 1 in 20 or 1 in 40

RESULTS

Figure 1 shows line immunoelectrophoresis of VVA HeLa against four different antisera diluted 1 in 20

Antiserum D is the reference antiserum obtained by the inoculation of rabbits with VVA rabbit lung fibroblasts as described under Methods. A total of 6 lines can be discerned The two upper lines have in other systems (cf (5)) been identified as the so-called

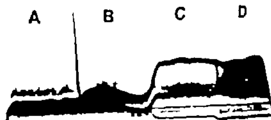


Fig 1 Line immunoelectrophoresis of different antisera against VVA HeLa.

A. Antiserum against the S immunoprecipitate obtained with antiserum C.

B. Antiserum against the L immunoprecipitate obtained with antiserum C.

C. Antiserum against the L and S immunoprecipitates from D

D Antiserum against whole virus.

The antisera were used in dilution 1 in 20.

L (or 10S) and the S (or 7S) antigens (*vide infra*)

Antiserum C was obtained by immunization with the L and S immunoprecipitates cut out from D The L and S precipitation lines were now better separated, and one or two of the other lines are missing

Antiserum B was produced by immunization with the L (lower) immunoprecipitate from antiserum C. A divalent antiserum was obtained giving the L and S precipitation lines only The antisera had become more potent with regard to L and S antibodies as the lines were lowered considerably (the antisera were used at the same dilution)

Antiserum A was produced by immunization with the S (upper) immunoprecipitate obtained with VVA HeLa and antiserum C. A monovalent anti-S antiserum was here obtained. The identity of the precipitation line was established by crossed line immunoelectrophoresis (Fig 2) The line produced by antiserum A shows a reaction of identity with the cathodic peak obtained with antiserum D In earlier experiments the more cathodic peak has been shown to be produced by the S (or 7S) antigen and the anodic one by the L antigen (5)



Fig 2 Crossed line immunoelectrophoresis showing reaction of identity between the S immunoprecipitate (cathodic) in D and the band in A. A and D Cf. legend to Fig. 1
The antisera were used in dilution 1 in 20.

When antiserum B was used more diluted (1 in 40) the L and S lines became well separated (Fig. 3) In this way it was possible to obtain an L immunoprecipitate which was not contaminated with the S antigen. The resulting antiserum (E in Fig. 3) gave a line which showed reaction of identity with the L (anodic or 10S) line produced with antiserum B. The anti-S (7S) and anti-L (10S) antisera were specific and of high potency A control antigen made from uninfected HeLa cell cultures produced no line using the antisera.

DISCUSSION

Several difficulties are met with when antisera against viral antigens are to be prepared

One major problem is to get rid of contaminating host cell antigenic material. In the case of vaccinia antigens this problem was in



Fig 3 Line and crossed line immunoelectrophoresis showing reaction of identity between the S (cathodic 7S) line of antiserum B with the line of antiserum A, and between the L (anodic 10S) line of antiserum B and the line of antiserum E. Antiserum E was produced by immunization with the L immunoprecipitate obtained by antiserum B.
The antisera were used in dilution 1 in 40

the present experiments circumvented by growing the virus in cells from the animal species going to be used for immunization. In the present work, vaccinia virus was grown in primary rabbit lung fibroblast cell cultures before being inoculated into rabbits. The cells were grown in a medium supplemented with serum from the very rabbit going to be used for immunization. Furthermore the antigen used for electrophoresis was prepared in a human cell line (HeLa) which was chosen because of high yields of virus.

The other major problem is to obtain an adequate purification of the antigen in question. This is usually accomplished through a series of separation procedures based on charge, size and density of the antigen. A short cut can be made when distinct and separated immunoprecipitates are produced in gels. It appeared in the present study that the immunoprecipitates may be contaminated by other antigenic materials. The final monovalent sera were a result of a three- and four step immunization series. The first immunizations were performed to produce an antiserum containing precipitating antibodies to L and S antigens only (serum D C and B) The eventual monovalent antisera, antisera A and E, were obtained by immunization with isolated S and L immunoprecipitates. A pure anti-S was easy to produce as might be expected from the precipitation pattern, since the S precipitate is the most anodic one The anti L antiserum was in one experiment contaminated with S precipitins. With other strains of vaccinia virus the L precipitate has been shown to be the more anodic one (unpublished data)

Immunization with isolated immunoprecipitates is a simple and efficient procedure for obtaining potent monospecific antisera against viral antigens.

REFERENCES

1 *Axelsen, N H., Krøll, J & Weeke B* (Eds.) A manual of quantitative immunoelectrophoresis. Methods and applications. Scand. J Immunol. 2 Suppl. 1 1973

2 *Harboe M Closs O & Deverill J* Production of monospecific antisera against antigenic components of *Mycobacterium bovis* (BCG) In press Scand. J Immunol

3 *Laurell C B* Antigen-antibody crossed electrophoresis. Analyt. Biochem. *10* 358–361 1965

4 *Nansen P Flagstad T & Pedersen K B* Preparation of antisera to bovine immunoglobulin classes by immunization with agar-gel precipitates. Acta path. microbiol. scand. Sect B, *79* 459–465 1971

5 *Prente P H., Heukenes G & Gerra, I* Identification of antigens within the so-called vaccinia L-S complex by means of quantitative immunoelectrophoresis. Acta path. microbiol. scand. Sect. B *84* 293–299 1976.

6 *Shivers C A & James J M.* Specific antibodies produced against antigens of agar-gel precipitates. Immunology *13* 547–554, 1967

7 *Vestergaard B F* Production of antiserum against a specific herpes simplex virus type 2 antigen. J Immunol. *4* Suppl. 2, 203–206, 1975

Acta path. microbiol. scand. Sect. C, 85 301–306 1977

THERMOSTABLE *NEISSERIA GONORRHOEAE* ANTIGENS EXAMINED BY A BACTERIAL AGGLUTINATION TEST

Guri Eggset Angelsen and Johan Mæland

Trondheim Regional Hospital, Department of Microbiology
University of Trondheim, 7000 Trondheim, Norway

Angelsen, G. Eggset & Mæland, J. Thermostable *Neisseria gonorrhoeae* antigens examined by bacterial agglutination test. Acta path. microbiol. scand. Sect. C, 85 301–306, 1977

Rabbit antisera against three different *N. gonorrhoeae* isolates agglutinated heated gonococci (100° C, 2 h) before and after treatment with periodate or pronase, but this was not the case with gonococci exposed to the combined action of the reagents. All agglutinins could be removed by absorption of antiserum with untreated or heat-treated gonococci or with a heat extract of the bacteria. Antiserum absorbed with the lipopolysaccharide still agglutinated the heated gonococci both before and after exposure of the bacteria to periodate or pronase. The results of cross-absorption experiments indicated strain variation of thermostable antigenic determinants involved in the agglutination reaction.

Key words: *Neisseria gonorrhoeae*; thermostable antigens; agglutination test.

J. Mæland, Department of Microbiology, Regional Hospital, N-7000 Trondheim, Norway

Received 17.ii.77 Accepted 25.iii.77

Many different techniques have been used for the study of *N. gonorrhoeae* antigens and their interaction with antibody (2, 12). The bacterial agglutination test was used by some of the earlier investigators, and several authors reported that this technique could be adopted for the detection of serotypes among gonococci (5, 14, 16). During recent years, investigators have used the co-agglutination test to identify gonococci (3), but only few have used the bacterial agglutination test. This may appear remarkable in view of the significant role of bacterial agglutination in serotyping of bacteria, including *N. meningitidis*, and in the sero-diagnosis of infectious diseases. A likely explanation for this could be that *N. gonorrhoeae* strains may show hyperagglutinability or autoagglutinability which may interfere with the agglutination reaction (16).

A bacterial agglutination test was used by us to study agglutinogens of heat-treated *N. gonorrhoeae* cells.

MATERIALS AND METHODS

Bacterial Strains

The *N. gonorrhoeae* strains 8551, V and VII used in this study have been described earlier (11). When examined according to *Kellogg et al.* (8) all three strains were found to be colony type T4.

Culture

The fermentation medium described by *Walhar & Flynn* (15) supplemented with 1 per cent glucose was used for cultivation of the gonococci.

Cultures were incubated in candle extinction jars at 37 C for 48 h. The bacteria were harvested in phosphate buffered saline pH 7 2 (PBS) and collected by centrifugation at 8000 × *g for 15 min.*

Treatment of Bacteria

Lipopolysacchar de (LPS) was prepared from whole gonococcal cells by extraction with phenol-water purification by ultracentrifugation and washing, and lyophilization (9)

Gonococci for use as antigen in the bacterial agglutination test were suspended in PBS, heated in a boiling water bath for 2 h and spun at 8000 × *g for 15 min. The supernatant (heat extract)* was dialysed against water for two days and lyophilized The heattreated bacteria were washed twice in PBS and kept at 4 C in PBS containing 0 02 per cent Na-azide The density of the bacterial suspension used in the agglutination test was determined spectrophotometrically as described under RESULTS

Oxidation with Na-*meta*-periodate (Merck) was *performed with 0 1 g* sedimented heated and washed bacteria in 20 ml PBS containing 0.5 per cent of the oxidizing agent. The suspension was then kept in the dark at 4 C *for 20 h* and centrifuged The sediment was washed three times and resuspended in PBS containing Na-azide

Heated gonococci were digested with pronase (B *Grade Calbiochem) A* bacterial suspension was *prepared as before in PBS containing the enzyme* (0 1 mg/ml) The suspension was then incubated at 37 C for 4 h and the enzyme activity destroyed by heating the suspension at *100 C for 5 min* The bacteria were washed and resuspended in PBS containing Na-azide

Immunization

White rabbits weighing approximately 2 5 kg were immunized with untreated bacteria of the three *N gonorrhoeae* strains suspended in 0 85 per cent NaCl The density of the suspension was adjusted to an optical transmission of 10 per cent at *540 nm Each animal was* injected intravenously on three successive days each week over a period of four weeks with increasing doses of the bacterial suspension *from 0 5 to 1 5 ml.* The animals were bled seven days after the last injection. The antisera (anti-Gc-8551 V and VII) were stored at —20 C and heated at 56 C *for 30 min* before testing

Bacterial Agglutination Test

Agglutination of the bacteria was performed on glass plates (Carl Hecht *Germany Cat. No. 2417)* containing 12 circular excavations, diameter 16 mm, depth 1 75 mm. Twofold dilutions of the antisera were prepared in PBS Then 0 025 ml was transferred to the excavation, followed by the addition of an equal volume of the heat-treated gonococci. The plates were kept in wet chambers at 20° C, unless otherwise stated. The agglutination was read *in oblique light against* a dark background and the degree of clumping recorded as — to + + + Bacteria added to PBS or normal rabbit serum were included as control. The titre is defined as the reciprocal of the highest serum dilution showing agglutination.

Absorption of Antiserum

Portions of antiserum were absorbed with untreated gonococci, heated bacteria before and after oxidation with periodate or digestion with pronase, the heat extract of gonococci, or LPS. For the absorption 0 ? g of the bacteria, 10 mg of the heat extract, or 1 mg or more of the LPS preparation, were used per 0.1 ml of undiluted antiserum. Absorption was carried out at 20 C for 30 min and at 4 C for 20 h. The mixture was then centrifuged at 2000 × *g* for 15 min and the supernatant saved.

RESULTS

Heat treated *N gonorrhoeae* cells formed agglutinates when added to rabbit antiserum (Fig 1) Bacterial suspensions were optimal for the agglutination reaction when adjusted to a density giving *a transmission* of 20 per cent at 540 nm. This bacterial density was used in the experiments to be described. The agglutination reaction was carried out by incubation at different temperatures and the results were recorded after various lengths of time (Table 1) The rapidity at which agglutinates formed at 20 C almost equalled that at 37° C. At these temperatures maximum titre was obtained *after* 3 h. At 4° C the agglutinates formed at a slower rate. Incubation at 20 C for 3 h was used in experiments described below

TABLE 1 *Titre in the Bacterial Agglutination Test of Anti-Gc I Against Heated* N gonorrhoeae *Strain I Effect of Incubation at Different Temperatures and for Various Lengths of Time*

	Hours			
Temperature	1	2	3	4
4 C	< 8	16	32	128
20 C	32	64	256	256
37 C	3?	128	256	256

Highly reproducible results were obtained when the titres of antisera were determined repeatedly. No prozone phenomenon was observed. Autoagglutinability or hyperagglutinability of the *N. gonorrhoeae* strains was not observed. The titre was not affected by storage of the heat treated bacteria at 4 °C for at least six weeks.

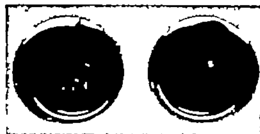


Fig. 1. Bacterial agglutination test with *N. gonorrhoeae* strain V added to anti-Gc V (left) and to normal rabbit serum (right) diluted 1:16.

Effect of Treatment of Gonococci with Periodate or Pronase

The morphology of the heated gonococci did not change during treatment with pronase in concentrations up to 0.1 mg per ml, or with periodate, as revealed by phase contrast microscopy. The bacterial cells were partially disintegrated when pronase in concentrations higher than 0.1 mg per ml was used.

Bacteria oxidized with periodate or digested with pronase were also agglutinated by antibody (Table 2). Oxidation of the bacteria with periodate followed by digestion with pronase resulted in abrogation of the agglutination, thus indicating destruction of the thermostable agglutinogens.

Effect of Absorption of Antisera with Various Materials of Gonococci

Antiserum against each of the *N. gonorrhoeae* strains was absorbed with various materials of the corresponding strain and similar results were obtained in each case. Antibodies against the heat-treated bacteria were removed when serum was absorbed with untreated gonococci, heated bacteria, or with the heat extract (Table 3). On the other hand, the antisera contained antibodies that could not be removed by absorption with periodate treated gonococci, as well as antibodies that resisted absorption with pronase-digested bacteria. Serum absorbed with periodate-treated and then with pronase-digested bacteria showed no antibody activity. Antiserum absorbed with LPS still agglutinated the untreated and treated gonococci. Identical results were obtained when increasing concentrations of LPS were used for absorption. The titres of antisera were not affected by absorption with gonococci exposed to the combined action of periodate and pronase. The results indicated that multiple antigens of various chemical nature are involved in the agglutination reaction.

Effect of Cross-Absorption of Antisera

Each of the antisera, anti-Gc-8551, V and VII agglutinated heated bacteria of all three strains before and after treatment with periodate or pronase. Antibody showing cross-reactivity with any one of the heterologous

TABLE 2. *Titre in the Bacterial Agglutination Test of Anti-Gc-8551, V and -VII against Heated Bacteria of the Homologous N. gonorrhoeae Strains. Effect of Treatment of Bacteria with Periodate, Pronase or both*

Antiserum	None	Bacteria treated with		
		Periodate	Pronase	Periodate + pronase
Anti-Gc-8551	128	128	64	< 8
Anti-Gc V	256	256	128	< 8
Anti-Gc VII	128	128	128	< 8

TABLE 3 *Titre in the Bacterial Agglutination Test of Anti-Gc V against Heated Bacteria of N gonorrhoeae Strain V before and after Treatment with Periodate or Pronase Effect of Absorption of Antiserum with various Materials of the same Strain*

Material used for absorption	Treatment of material before absorption	None	Bacteria treated with Periodate	Pronase
None		256	256	128
Bacteria	None	< 8	< 8	< 8
"	100 C, 2h	< 8	< 8	< 8
"	100 C, 2h + periodate	128	< 8	128
"	100 C, 2h + pronase	256	256	< 8
"	100 C 2h + periodate + pronase	256	256	128
Heat extract	None	< 8	< 8	< 8
LPS	"	128	128	128

TABLE 4 *Titre in the Bacterial Agglutination Test of Anti Gc V against Heated N gonorrhoeae Strain V Effect of Absorption of Serum with Homologous and Heterologous Strains*

Strain used for absorption	Bacteria treated with None	Periodate	Pronase
None	256	256	128
V	< 8	< 8	< 8
8551	128	64	64
VII	128	64	64

strains could be removed by absorption with this same strain However cross absorbed samples of any of the antisera still agglutinated untreated or treated bacteria of the homologous strain (Table 4) thus indicating strain variation of agglutinogens.

DISCUSSION

The agglutination test described was practical for the demonstration of antibody agglutinating heated *N gonorrhoeae* cells. However precise standardization of the technique was necessary to ensure adequate sensitivity and reproducibility of the test. The inagglutinability hyperagglutinability or autoagglutinability which have hampered agglutination reactions with *N gonorrhoeae cells* (16) were not observed with the strains used in the present study This might be due to the effect of heating of the bacteria (16) and/or the T4 colony type of the gonococci used (13) The antisera used in this study were raised in rabbits by immunization with untreated gonococcal cells. This means that only antigens preserved during heating of the bacteria participated in the agglutination reaction, not an artifact of the heat treatment.

After treatment of the heated gonococci with periodate or pronase antibody mediated agglutination was still observed, but this was not the case after exposure of the bacteria to both the reagents. Using absorbed serum specimens, it was further demonstrated that agglutinins combined with antigen(s) showing resistance to pronase and sensitivity to periodate and with antigen showing sensitivity to pronase and resistance to periodate. The results are in accordance with the assumption that thermostable agglutinogens of gonococci include two major antigens or groups of antigens, one sensitive to periodate and the other sensitive to pronase This is similar to previous observations on the endotoxin from *N gonorrhoeae* cells. Serum from immunized rabbits contained antibodies which combined with the carbohydrate component and antibodies

which combined with the protein component of the endotoxin complex (10). However the results of this study showed that antiserum absorbed with LPS of the homologous strain still agglutinated the bacteria both before and after treatment with periodate or pronase. This finding does not exclude the fact that antigenic determinants of the endotoxin complex might have participated in the agglutination reaction. However the results indicate that other thermostable antigens, probably of varying chemical nature, were also involved, as in agglutination by antibody of heat treated cells of *Pseudomonas aeruginosa* observed by others (4). The data in the present study are in accordance with recent observations by others to the effect that the outer membrane of the cell envelope of gonococci contains several components with potential immunogenicity (7, 17). With the exception of the thermostable antigenic determinants of the endotoxin (9), the susceptibility to heat treatment of other envelope antigens of gonococci or their role in antibody mediated agglutination of the bacteria is not known.

As recently shown by *Buchanan & Pearce* (1), piliated gonococci can be agglutinated by antibody to the pili antigen. With the strains used in this study, it can be excluded that pili-related antigens participated in the agglutination reaction, since it can be assumed that the type T4 cells of the strains used did not produce pili (6, 13).

Antisera absorbed with heterologous strains of gonococci still contained antibody which agglutinated untreated and treated bacteria of the homologous strain. This observation further emphasizes the complex mosaic of the antigens of heated gonococci, including antigen that may vary in specificity from one strain to another. It remains to be shown whether these antigens can form the basis necessary for the serotyping of gonococci.

Antibody activity was eliminated by absorption of antisera with the heat extract of the corresponding bacterium. Thus, it can be assumed that the various antigens involved in the bacterial agglutination reaction were present also in the heat extract. Analysis of the heat extract may provide an alternative approach to the study of these antigens.

This work was supported by grants from *The Norwegian Research Council for Science and the Humanities*.

REFERENCES

1. *Buchanan, T. M. & Pearce, W. A.*: Pili as mediator of the attachment of gonococci to human erythrocytes. Infect. Immunity *13*: 1483–1489, 1976.
2. *Cheng, A. F. & Kwapinski, J. B. G.*: Perspectives of the immunology of gonorrhoeae. Zbl. Bakt. Hyg. I. Abt. Ref. *245*: 481–518, 1975.
3. *Danielsson, D. & Kronvall, G.*: Slide agglutination method for the serological identification of *Neisseria gonorrhoeae* with anti-gonococcal antibodies adsorbed to protein A-containing staphylococci. Appl. Microbiol. *27*: 368–374, 1974.
4. *Duncan, N. H., Hinton, N. A., Penner, J. L. & Duncan, I. B. R.*: Preparation of typing antisera specific for O antigens of *Pseudomonas aeruginosa*. J. Clin. Microbiol. *4*: 124–128, 1976.
5. *Hermanies, J.*: Gonococcus types. I. J. Infect. Dis. *28*: 133–142, 1921.
6. *Jephcott, A. E., Reyn, A. & Birch-Andersen, A.*: *Neisseria gonorrhoeae*. III. Demonstration of presumed appendages to cells from different colony types. Acta path. microbiol. scand. Sect. B *79*: 437–439, 1971.
7. *Johnston, K. H. & Gotschlich, E. C.*: Isolation and characterization of the outer membrane of *Neisseria gonorrhoeae*. J. Bact. *119*: 250–257, 1974.
8. *Kellogg, D. S. J., Peacock, W. L. J., Deacon, W. E., Brown, L. & Pirkle, C. I.*: *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. J. Bact. *85*: 1274–1279, 1963.
9. *Maeland, J. A.*: Antigenic properties of various preparations of *Neisseria gonorrhoeae* endotoxin. Acta path. microbiol. scand. *73*: 413–422, 1968.
10. *Maeland, J. A.*: Antigenic determinants of aqueous ether extracted endotoxin from *Neisseria gonorrhoeae*. Acta path. microbiol. scand. *76*: 475–483, 1969.
11. *Maeland, J. A.*: Serological cross-reactions of aqueous ether extracted endotoxin from *Neisseria gonorrhoeae* strains. Acta path. microbiol. scand. *77*: 505–517, 1969.
12. *Sandström, E. & Danielsson, D.*: A survey of gonococcal serology. I. *Danielsson, D., Juhlin, L. & Mårdh, P. A.* (Eds.): Genital In-

TABLE 3 *Titre in the Bacterial Agglutination Test of Anti-Gc V against Heated Bacteria of* N gonorrhoeae *Strain V before and after Treatment with Periodate or Pronase Effect of Absorption of Antiserum with various Materials of the same Strain*

Material used for absorption	Treatment of material before absorption	None	Bacteria treated with Periodate	Bacteria treated with Pronase
None		256	256	128
Bacteria	None	< 8	< 8	< 8
"	100 C, 2h	< 8	< 8	< 8
"	100 C, 2h + periodate	128	< 8	128
"	100 C, 2h + pronase	256	256	< 8
"	100 C, 2h + periodate + pronase	256	256	128
Heat extract	None	< 8	< 8	< 8
LPS	"	128	128	128

TABLE 4 *Titre in the Bacterial Agglutination Test of Anti-Gc V against Heated N* gonorrhoeae *Strain V Effect of Absorption of Serum with Homologous and Heterologous Strains*

Strain used for absorption	Bacteria treated with None	Periodate	Pronase
None	256	256	128
V	< 8	< 8	< 8
8551	128	64	64
VII	128	64	64

strains could be removed by absorption with this same strain. However cross-absorbed samples of any of the antisera still agglutinated untreated or treated bacteria of the homologous strain (Table 4) thus indicating strain variation of agglutinogens.

DISCUSSION

The agglutination test described was practical for the demonstration of antibody agglutinating heated *N gonorrhoeae* cells. However precise standardization of the technique was necessary to ensure adequate sensitivity and reproducibility of the test The inagglutinability hyperagglutinability or autoagglutinability which have hampered agglutination reactions with *N gonorrhoeae cells* (16) were not observed with the strains used in the present study This might be due to the effect of heating of the bacteria (16) and/or the T4 colony type of the gonococci used (13) The antisera used in this study were raised in rabbits by immunization with untreated gonococcal cells. This means that only antigens preserved during heating of the bacteria participated in the agglutination reaction, not an artifact of the heat treatment.

After treatment of the heated gonococci with periodate or pronase antibody mediated agglutination was still observed but this was not the case after exposure of the bacteria to both the reagents. Using absorbed serum specimens, it was further demonstrated that agglutinins combined with antigen(s) showing resistance to pronase and sensitivity to periodate, and with antigen showing sensitivity to pronase and resistance to periodate. The results are in accordance with the assumption that thermostable agglutinogens of gonococci include two major antigens or groups of antigens, one sensitive to periodate and the other sensitive to pronase. This is similar to previous observations on the endotoxin from *N gonorrhoeae* cells. Serum from immunized rabbits contained antibodies which combined with the carbohydrate component and antibodies

which combined with the protein component of the endotoxin complex (10) However the results of this study showed that antiserum absorbed with LPS of the homologous strain still agglutinated the bacteria both before and after treatment with periodate or pronase. This finding does not exclude the fact that antigenic determinants of the endotoxin complex might have participated in the agglutination reaction. However the results indicate that other thermostable antigens, probably of varying chemical nature, were also involved, as in agglutination by antibody of heat-treated cells of *Pseudomonas aeruginosa* observed by others (4) The data in the present study are in accordance with recent observations by others to the effect that the outer membrane of the cell envelope of gonococci contains several components with potential immunogenicity (7 17) With the exception of the thermostable antigenic determinants of the endotoxin (9) the susceptibility to heat treatment of other envelope antigens of gonococci or their role in antibody mediated agglutination of the bacteria is not known.

As recently shown by *Buchanan & Pearce* (1) piliated gonococci can be agglutinated by antibody to the pili antigen. With the strains used in this study it can be excluded that pili related antigens participated in the agglutination reaction, since it can be assumed that the type T4 cells of the strains used did not produce pili (6, 13)

Antisera absorbed with heterologous strains of gonococci still contained antibody which agglutinated untreated and treated bacteria of the homologous strain. This observation further emphasizes the complex mosaic of the antigens of heated gonococci, including antigen that may vary in specificity from one strain to another It remains to be shown whether these antigens can form the basis necessary for the serotyping of gonococci.

Antibody activity was eliminated by absorption of antisera with the heat extract of the corresponding bacterium Thus, it can be assumed that the various antigens involved in the bacterial agglutination reaction were present also in the heat extract. Analysis of the heat extract may provide an alternative approach to the study of these antigens.

This work was supported by grants from *The Norwegian Research Council for Science and the Humanities.*

REFERENCES

1 *Buchanan R. M & Pearce W A.* Pili as mediator of the attachment of gonococci to human erythrocytes. Infect. Immunity *13* 1483–1489 1976
2. *Cheng, A. F & Kwapinski J B. G.:* Perspectives of the immunology of gonorrhoeae. Zbl. Bakt. Hyg I. Abt. Ref. *245* 481 518 1975
3 *Danielsson D & Kronvall, G* Slide agglutination method for the serological identification of *Neisseria gonorrhoeae* with anti-gonococcal antibodies adsorbed to protein A-containing staphylococci. Appl. Microbiol. *27* 368–374 1974
4 *D can N H., Hinton N A., Penner J L. & Duncan I B. R.:* Preparation of typing antisera specific for O antigens of *Pseudomonas aeruginosa.* J clin. Microbiol. *4* 124–128, 1976
5 *Hermanies, J..* Gonococcus types I J Infect. Dis. *28* 133–142, 1921
6. *J phcott A. E Rey A. & Birch-Andersen A.* *Neisseria gonorrhoeae* III Demonstration of presumed appendages to cells from different colony types Acta path. microbiol. scand Sect. B, *79* 437–439 1971
7 *Johnston K. H & Gotschlich E. C* Isolation and characterization of the outer membrane of *Neisseria gonorrhoeae.* J Bact. *119* 250–257 1974
8. *Kellogg D S J Peacock, W L. J Deacon W E., Brown L. & Pirkle C I* *Neisseria gonorrhoeae* I Virulence genetically linked to clonal variation. J Bact. *85* 1274–1279 1963
9 *Maland J A.:* Antigenic properties of various preparations of *Neisseria gonorrhoeae* endotoxin. Acta path. microbiol. scand. *73* 413 422, 1968.
10. *Maland J A* Antigenic determinants of aqueous ether extracted endotoxin from *Neisseria gonorrhoeae* Acta path. microbiol. scand. *76* 475–483 1969
11 *Maland J A* Serological cross-reactions of aqueous ether extracted endotoxin from *Neisseria gonorrhoeae* strains. Acta path. microbiol. scand. *77* 303–317 1969
12. *Sandstrom, E. & Danielsson D* A survey of gonococcal serology In *Danielsson D J k-Eu, L. & Mårdh P A.* (Eds.) Genital in-

TABLE 3 *Titre in the Bacterial Agglutination Test of Anti-Gc V against Heated Bacteria of N gonorrhoeae Strain V before and after Treatment with Periodate or Pronase Effect of Absorption of Antiserum with various Materials of the same Strain*

Material used for absorption	Treatment of material before absorption	None	Bacteria treated with	
			Periodate	Pronase
None		256	256	128
Bacteria	None	< 8	< 8	< 8
"	100 C 2h	< 8	< 8	< 8
"	100 C 2h + periodate	128	< 8	128
"	100 C 2h + pronase	256	256	< 8
"	100 C 2h + periodate + pronase	256	256	128
Heat extract	None	< 8	< 8	< 8
LPS	"	128	128	128

TABLE 4 *Titre in the Bacterial Agglutination Test of Anti-Gc V against Heated N gonorrhoeae Strain V Effect of Absorption of Serum with Homologous and Heterologous Strains*

Strain used for absorption	Bacteria treated with		
	None	Periodate	Pronase
None	256	256	128
V	< 8	< 8	< 8
8551	128	64	64
VII	128	64	64

strains could be removed by absorption with this same strain. However cross-absorbed samples of any of the antisera still agglutinated untreated or treated bacteria of the homologous strain (Table 4) thus indicating strain variation of agglutinogens

DISCUSSION

The agglutination test described was practical for the demonstration of antibody agglutinating heated *N gonorrhoeae* cells. However precise standardization of the technique was necessary to ensure adequate sensitivity and reproducibility of the test. The inagglutinability hyperagglutinability or autoagglutinability which have hampered agglutination reactions with *N gonorrhoeae cells* (16) were not observed with the strains used in the present study This might be due to the effect of heating of the bacteria (16) and/or the T4 colony type of the gonococci used (13) The antisera used in this study were raised in rabbits by immunization with untreated gonococcal cells. This means that only antigens preserved during heating of the bacteria participated in the agglutination reaction, not an artifact of the heat treatment.

After treatment of the heated gonococci with periodate or pronase antibody mediated agglutination was still observed but this was not the case after exposure of the bacteria to both the reagents. Using absorbed serum specimens it was further demonstrated that agglutinins combined with antigen(s) showing resistance to pronase and sensitivity to periodate and with antigen showing sensitivity to pronase and resistance to periodate. The results are in accordance with the assumption that thermostable agglutinogens of gonococci include two major antigens or groups of antigens, one sensitive to periodate and the other sensitive to pronase. This is similar to previous observations on the endotoxin from *N gonorrhoeae cells* Serum from immunized rabbits contained antibodies which combined with the carbohydrate component and antibodies

which combined with the protein component of the endotoxin complex (10) However the results of this study showed that antiserum absorbed with LPS of the homologous strain still agglutinated the bacteria both before and after treatment with periodate or pronase. This finding does not exclude the fact that antigenic determinants of the endotoxin complex might have participated in the agglutination reaction. However the results indicate that other thermostable antigens, probably of varying chemical nature, were also involved as in agglutination by antibody of heat-treated cells of *Pseudomonas aeruginosa* observed by others (4) The data in the present study are in accordance with recent observations by others to the effect that the outer membrane of the cell envelope of gonococci contains several components with potential immunogenicity (7 17) With the exception of the thermostable antigenic determinants of the endotoxin (9) the susceptibility to heat treatment of other envelope antigens of gonococci or their role in antibody-mediated agglutination of the bacteria is not known.

As recently shown by *Buchanan & Pearce* (1) piliated gonococci can be agglutinated by antibody to the pili antigen. With the strains used in this study it can be excluded that pili-related antigens participated in the agglutination reaction, since it can be assumed that the type T4 cells of the strains used did not produce pili (6, 13)

Antisera absorbed with heterologous strains of gonococci still contained antibody which agglutinated untreated and treated bacteria of the homologous strain. This observation further emphasizes the complex mosaic of the antigens of heated gonococci, including antigen that may vary in specificity from one strain to another It remains to be shown whether these antigens can form the basis necessary for the serotyping of gonococci.

Antibody activity was eliminated by absorption of antisera with the heat extract of the corresponding bacterium. Thus, it can be assumed that the various antigens involved in the bacterial agglutination reaction were present also in the heat extract. Analysis of the heat extract may provide an alternative approach to the study of these antigens.

This work was supported by grants from *The Norwegian Research Council for Science and the Humanities.*

REFERENCES

1 *Buchanan R. M & Pearce W. A* Pili as mediator of the attachment of gonococci to human erythrocytes. Infect. Immunity *13* 1483–1489 1976

2 *Cheng, A. F & Kasprowski J B G.* Perspectives of the immunology of gonorrhoeae Zbl. Bakt. Hyg I. Abt. Ref. *245* 481–518, 1975

3 *Danielsson D & Kronvall G* Slide agglutination method for the serological identification of *Neisseria gonorrhoeae* with anti-gonococcal antibodies adsorbed to protein A-containing staphylococci. Appl. Microbiol. *27* 368–374 1974

4 *Duncan N H Hinton N A., Penner J L. & Duncan, I B. R.:* Preparation of typing antisera specific for O antigens of *Pseudomonas aeruginosa.* J clin. Microbiol. *4* 124–128, 1976

5. *Hermanies, J* Gonococcus types I J Infect. Dis. *28* 133–142, 1921

6 *Jephcott A E., Reyn A. & Birch-Andersen A.. Neisseria gonorrhoeae* III Demonstration of presumed appendages to cells from different colony types Acta path. microbiol. scand Sect. B, *79* 437–439 1971

7 *Johnston, K H & Gotschlich E. C* Isolation and characterization of the outer membrane of *Neisseria gonorrhoeae* J Bact. *119* 250–257 1974

8. *Kellogg D S J Peacock W L. J Deacon W E., Brown L. & Pirkle C I Neisseria gonorrhoeae* I. Virulence genetically linked to clonal variation. J Bact. *85* 1274–1279 1963

9 *Maeland J A* Antigenic properties of various preparations of *Neisseria gonorrhoeae* endotoxin. Acta path. microbiol. scand. *73* 413 422, 1968.

10 *Maeland J A..* Antigenic determinants of aqueous ether extracted endotoxin from *Neisseria gonorrhoeae* Acta path. microbiol. scand. *76* 475–483 1969

11 *Maeland J A* Serological cross-reactions of aqueous ether extracted endotoxin from *Neisseria gonorrhoeae* strains. Acta path. microbiol scand. *77* 505–517 1969

12. *Sandstrom E. & Danielsson D* A survey of gonococcal serology In: *Danielsson D Juhlin L. & Mårdh P A.* (Eds.) Genital in-

fections and their complications. Almquist & Wiksell International Stockholm, 1975 p 253–259

13 *Swanson J Kraus S J & Gotschlich E. C.*. Studies on gonococcus infection I Pili and zones of adhesion Their relation to gonococcal growth patterns. J exp. Med. *134* 886–906 1971

14 *Tulloch W J* Serological examination of one hundred strains of the gonococcus. J Path. Bact. *25* 346–365 1922.

15 *Watkins S A & Flynn J* A serum-free medium for testing fermentation reactions in *Neisseria gonorrhoeae* J clin. Path. 25 525-527 1972.

16 *Wilson J F*. A serological study of *Neisseria gonorrhoeae* J Path. Bact. 68 495–510, 1954

17 *Wolf Watz H Elmros T., Normark, S & Blom G* Cell envelope of *Neisseria gonorrhoeae* Outer membrane and peptidoglycan composition of penicillin-sensitive and -resistant strains. Infect. Immunity *11* 1332 1341, 1975

Acta path. microbiol. scand. Sect. C, 85: 307–313, 1977

IN VITRO EFFECTS OF RUBELLA VIRUS, STRAIN RA 27/3, ON HUMAN LYMPHOCYTES

II Specific Antigen Stimulation in Relation to Rubella Haemagglutination Inhibition Antibodies

ROLF MALLER and LARS SÖRÉN

Department of Clinical Bacteriology and Department of Infectious Diseases, Linköping University S-581 85 Linköping, Sweden

Maller R. & Sörén, L. *In vitro* effects of rubella virus, strain RA 27/3 on human lymphocytes. II. Specific antigen stimulation in relation to rubella haemagglutination inhibition antibodies. Acta path. microbiol. scand. Sect. C, 85 307–313 1977

Investigation has been made of the specific *in vitro* stimulation of human lymphocytes by live or inactivated rubella virus of the strain RA 27/3. The thymidine incorporation of lymphocytes from blood donors with different serological immunity against rubella was measured following incubation with the viral antigens. UV-inactivated rubella virus caused a moderate degree of stimulation of lymphocytes from seropositive individuals. On the other hand, live rubella virus stimulated lymphocytes from donors with low titres of anti-rubella HI-antibodies but not lymphocytes from donors with high antibody titres. Possible explanations for this discrepancy are discussed.

Key words: Rubella virus; lymphocytes *in vitro*; antigen specific stimulation; haemagglutination inhibition antibodies.

R. Maller Regionsjukhuset, Linköping, S-581-85 Linköping, Sweden.

Received 18.ii.77 Accepted 22.iii.77

Lymphocytes from rubella-immune donors can be activated to proliferate *in vitro* if cultured in the presence of inactivated rubella virus (9 16, 17 22, 23) This is true of several other viruses (1 3 4, 12, 13 15 21) and in general, preparations of inactivated virus are more powerful stimulants in specific lymphocyte stimulation than live virus (13) It is known, in fact, that many live viruses (10) including rubella virus (8, 11 14 16) can actually inhibit lymphocyte proliferation induced by mitogens or antigens *in vitro* In the case of rubella virus, this inhibition is correlated with the immune status of the lymphocyte donor against rubella, since pronounced inhibition of the PHA response was observed with lymphocytes from donors with high titres of antirubella antibodies, whereas there was no inhibition when the donors lacked serological immunity against rubella (8) The relationship between the two effects of rubella virus *in vitro*—specific lymphocyte stimulation and specific inhibition—and factors determining which effect will predominate, are not known at present. This paper presents a study of specific lymphocyte stimulation by live and UV-inactivated rubella virus in relation to serological immunity of the lymphocyte donor against rubella.

MATERIALS AND METHODS

Lymphocyte donors: Healthy volunteers, eighteen men and six women, aged 19–58 years (average 32 years) were used as lymphocyte donors. The rubella

fections and their complications. Almquist & Wiksell International Stockholm, 1975 p 253–259

13 *Swanson J Kraus S J & Gotschlich E. C* Studies on gonococcus infection I Pili and zones of adhesion Their relation to gonococcal growth patterns. J exp Med. *134* 886–906 1971

14 *Tulloch W J* Serological examination of one hundred strains of the gonococcus. J Path. Bact. *25* 346–365 1922

15 *Watkins S A & Flynn, J* A serum-free medium for testing fermentation reactions in *Neisseria gonorrhoeae* J clin. Path. 25 525–527 1972.

16 *Wilson J F.* A serological study of *Neisseria gonorrhoeae* J Path. Bact. *68* 495–510 1954

17 *Wolf Watz H Elmros T Normark S & Blom G* Cell envelope of *Neisseria gonorrhoeae* Outer membrane and peptidoglycan composition of penicillin-sensitive and -resistant strains. Infect. Immunity *11* 1332–1341, 1975

Acta path. microbiol. scand. Sect. C, 85: 307–313, 1977

IN VITRO EFFECTS OF RUBELLA VIRUS, STRAIN RA 27/3, ON HUMAN LYMPHOCYTES

II. Specific Antigen Stimulation in Relation to Rubella Haemagglutination Inhibition Antibodies

ROLF MALLER and LARS SÖRÉN

Department of Clinical Bacteriology and Department of Infectious Diseases, Linköping University, S-581 85 Linköping, Sweden

Maller, R. & Sörén, L. *In vitro* effects of rubella virus, strain RA 27/3, on human lymphocytes. II. Specific antigen stimulation in relation to rubella haemagglutination inhibition antibodies. Acta path. microbiol. scand. Sect. C, 85: 307–313, 1977.

Investigation has been made of the specific *in vitro* stimulation of human lymphocytes by live or inactivated rubella virus of the strain RA 27/3. The thymidine incorporation of lymphocytes from blood donors with different serological immunity against rubella was measured following incubation with the viral antigens. UV-inactivated rubella virus caused a moderate degree of stimulation of lymphocytes from seropositive individuals. On the other hand, live rubella virus stimulated lymphocytes from donors with low titres of anti-rubella HI-antibodies but not lymphocytes from donors with high antibody titres. Possible explanations for this discrepancy are discussed.

Key words: Rubella virus; lymphocytes; *in vitro*; antigen specific stimulation; haemagglutination inhibition antibodies.

R. Maller, *Regionsjukhuset, Linköping, S-581 85 Linköping, Sweden.*

Received 18.ii.77. Accepted 22.iii.77.

Lymphocytes from rubella immune donors can be activated to proliferate *in vitro* if cultured in the presence of inactivated rubella virus (9, 16, 17, 22, 23). This is true of several other viruses (1, 3, 4, 12, 13, 15, 21) and in general, preparations of inactivated virus are more powerful stimulants in specific lymphocyte stimulation than live virus (13). It is known, in fact, that many live viruses (10) including rubella virus (8, 11, 14, 16) can actually inhibit lymphocyte proliferation induced by mitogens or antigens *in vitro*. In the case of rubella virus, this inhibition is correlated with the immune status of the lymphocyte donor against rubella, since pronounced inhibition of the PHA response was observed with lymphocytes from donors with high titres of antirubella antibodies, whereas there was no inhibition when the donors lacked serological immunity against rubella (8). The relationship between the two effects of rubella virus *in vitro*—specific lymphocyte stimulation and specific inhibition—and factors determining which effect will predominate, are not known at present. This paper presents a study of specific lymphocyte stimulation by live and UV-inactivated rubella virus in relation to serological immunity of the lymphocyte donor against rubella.

MATERIALS AND METHODS

Lymphocyte donors: Healthy volunteers, eighteen men and six women, aged 19–58 years (average 32 years) were used as lymphocyte donors. The rubella

haemagglutination inhibiting (HI) antibodies in their sera were determined by the standard method used in our laboratory (2). Each HI figure was set from the results of three separate determinations.

In the serological testing performed at the Department of Clinical Bacteriology, Linköping, about half of those with serological immunity have titres above 40 and the other half titres between 5 and 20. Therefore, the donors in the present investigation were divided into three groups:

Group A (9 persons): moderate to high titres, HI $\geq$ 40. Group B (7 persons): low titres, HI = 5–20, and Group C (8 persons): no serological immunity, HI $<$ 5.

Rubella virus. Attenuated rubella virus of strain RA 27/3 was kindly provided by Dr *Pertti I. Mäkinen*, Orion Diagnostica, Helsinki, Finland. The preparation of the virus has been described previously (8, 20). However, in this investigation a final Millipore filtration was included (19).

The infectivity of the virus preparation was about 10^7 pfu/ml. The virus was suspended in "virus buffer" (0.025 M phosphate buffer pH 7.3 with EDTA 0.003 M), which was also used for the viral dilutions.

When applicable, the virus was UV-inactivated in a quartz vessel for 10 min at 254 nm (radiation energy obtained in the vessel = 480 μW/cm²). Under these conditions rubella virus is inactivated without losing its antigenic properties (5). "Cell control" solution was prepared from mock infected BHK/13S cells.

Lymphocyte separation and lymphocyte cultures. Lymphocytes were separated from newly-drawn venous blood, as described previously (8). The lymphocytes were washed three times in TCM 199 and suspended in TCM 199 with 15 per cent heat inactivated homologous rubella antibody-free AB-serum (HS) in a concentration of 0.7 $\times$ 10^6 mononuclear cells/ml. The final cell suspensions contained about 90 per cent mononuclear cells, and the cell viability, confirmed by trypan blue exclusion, was well above 90 per cent.

Rubella virus, "cell control" or "virus buffer" in 0.2 ml aliquots were added to 1.8 ml lymphocyte suspensions in roundbottom glass tubes with screw caps and incubated at 37° C in humidified air for 7 days. All cultures were performed in duplicate. Eighteen hours before the end of the incubation period, 0.15 μCi ³H-TdR in 0.1 ml of saline was added to each culture. Three samples of 0.5 ml from each culture were collected on glass fibre filters using a multiple sample processor (3025 Sampling Manifold, Millipore Co., Bedford, Massachusetts, USA). The cells were washed with physiological saline and 5 per cent trichloroacetic acid. The filters were placed in 10 ml Permablend III (Packard Instrument Co., Ill., USA) and the radioactivity was measured in a Packard Tri-Carb 3375 scintillation counter. The mean of the three samples from each tube was determined, and finally the mean of adequate duplicates was calculated and expressed as cpm/ml of cell suspension.

Statistics. The statistical significance for differences between different groups was checked using the Student's t-test.

RESULTS

The mean thymidine incorporation for cultures incubated with live rubella virus (L-RV), UV inactivated rubella virus (UV RV) and cell control (CC) in different dilutions, as well as virus buffer (VB), is shown in Fig. 1. In the group with no serological immunity to rubella (group C), the mean thymidine incorporation is the same for lymphocytes grown in the presence of L-RV, UV RV, CC and VB and is constant over the whole range of antigen dilutions (Fig. 1 C). In the group with low HI titres (group B), the mean thymidine incorporation of cells cultured with CC is also fairly constant at different antigen concentrations and similar to that of cells incubated with VB. Following incubation with both L-RV and UV RV, the incorporation increases with increasing antigen concentration (Fig. 1 B). A similar increase is seen in the cultures incubated with UV RV in the group with high HI-titres (group A) (Fig. 1 A). However, in this group no increase is seen in cells incubated with L-RV, the mean thymidine incorporation of these cultures being quite similar to that of cultures incubated with CC.

In most experiments, the antigen concentration for optimal stimulation was 10^6 pfu/ml. However, this could vary and in some experiments it was as low as 10^4 pfu/ml. Tables 1–3 show the stimulation obtained in individual experiments with groups A–C following challenge by antigen in a concentration giving optimal activation by UV RV in each experiment. In these tables the stimulation is expressed as 1) *specific stimulation*, i.e. the difference between thymidine incorporation of cells incubated with virus (L-RV or UV RV) and that of cells incubated with CC, and 2) *lymphocyte stimulation index (LSI)*, i.e. the thymidine incorporation of cells grown with

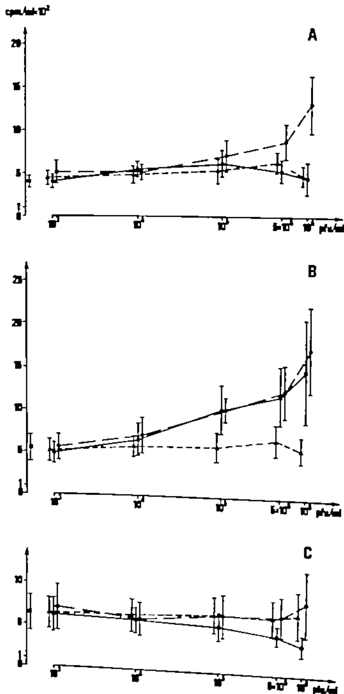


Fig. 1 Thymidine incorporation of lymphocytes incubated with live rubella virus (L-RV) UV-inactivated rubella virus (UV RV) and cell control (CC) in different dilutions.

A. Lymphocyte donors with HI titres ranging from 40 to 160 (n = 9)

B. Lymphocyte donors with HI titres ranging from 5 to 20 (n = 7)

C. Lymphocyte donors with HI titres less than 5 (n = 8)

Symbols

● – – –● UV RV

○——○ L-RV

△- - - -△ CC

■ virus buffer

Vertical bars denote standard error of the mean.

virus divided by that of cells grown with CC. The former values were used for statistical calculations.

After incubation with UV RV no lymphocyte activation is seen in the experiments with group C, all the LSI are about 1 (Table 3) However in the experiments with groups A and B, a variable degree of stimulation is ev

haemagglutination inhibiting (HI) antibodies in their sera were determined by the standard method used in our laboratory (2) Each HI figure was set from the results of three separate determinations.

In the serological testing performed at the Department of Clinical Bacteriology Linköping about half of those with serological immunity have titres above 40 and the other half titres between 5 and 20 Therefore the donors in the present investigation were divided into three groups

Group A (9 persons) moderate to high titres, HI $\geq$ 40 Group B (7 persons) low titres, HI = 5–20 and Group C (8 persons) no serological immunity HI $<$ 5

Rubella virus Attenuated rubella virus of strain RA 27/3 was kindly provided by Dr *Pertti Väänänen* Orion Diagnostica, Helsinki Finland. The preparation of the virus has been described previously (8 20) However in this investigation a final Millipore filtration was included (19)

The infectivity of the virus preparation was about 10^7 pfu/ml The virus was suspended in "virus buffer" (0.025 M phosphate buffer pH 7.3 with EDTA 0 005 M) which was also used for the viral dilutions.

When applicable the virus was UC-inactivated in a quartz vessel for 10 min at 254 nm (radiation energy obtained in the vessel = 480 μW/cm²) Under these conditions rubella virus is inactivated without losing its antigenic properties (5) "Cell control" solution was prepared from mock-infected BHK/13S cells.

Lymphocyte separation and lymphocyte cultures Lymphocytes were separated from newly-drawn venous blood as described previously (8) The lymphocytes were washed three times in TCM 199 and suspended in TCM 199 with 15 per cent heat inactivated homologous rubella antibody free AB-serum (HS) in a concentration of 0 7 $\times$ 10^6 mononuclear cells/ml. The final cell suspensions contained about 90 per cent mononuclear cells and the cell viability confirmed by trypan blue exclusion, was well above 90 per cent.

Rubella virus, "cell control or "virus buffer" in 0.2 ml aliquots were added to 1 8 ml lymphocyte suspensions in roundbottom glass tubes with screw caps and incubated at 37 C in humidified air for 7 days. All cultures were performed in duplicate. Eighteen hours before the end of the incubation period 0 15 μCi ^{14}C-TdR in 0.1 ml of saline was added to each culture. Three samples of 0 5 ml from each culture were collected on glass fibre filters, using a multiple sample processor (3075 Sampling Manifold, Millipore Co., Bedford Massachusets, USA) The cells were washed with physiological saline and 5 per cent trichloroacetic acid. The filters were placed in 10 ml Permablend III (Packard Instrument Co., Ill., USA) and the radioactivity was measured in a Packard Tri-Carb 3375 scintillation counter The mean of the three samples from each tube was determined, and finally the mean of adequate duplicates was calculated and expressed as cpm/ml of cell suspension.

Statistics The statistical significance for differences between different groups was checked using the Student s t test.

RESULTS

The mean thymidine incorporation for cultures incubated with live rubella virus (L-RV) UV inactivated rubella virus (UV RV) and cell control (CC) in different dilutions, as well as virus buffer (VB) is shown in Fig 1 In the group with no serological immunity to rubella (group C) the mean thymidine incorporation is the same for lymphocytes grown in the presence of L-RV UV RV CC and VB and is constant over the whole range of antigen dilutions (Fig 1 C) In the group with low HI titres (group B) the mean thymidine incorporation of cells cultured with CC is also fairly constant at different antigen concentrations and similar to that of cells incubated with VB Following incubation with both L-RV and UV RV the incorporation increases with increasing antigen concentration (Fig 1 B) A similar increase is seen in the cultures incubated with UV RV in the group with high HI titres (group A) (Fig 1 A) However in this group no increase is seen in cells incubated with L-RV the mean thymidine incorporation of these cultures being quite similar to that of cultures incubated with CC.

In most experiments, the antigen concentration for optimal stimulation was 10^6 pfu/ml. However this could vary and in some experiments it was as low as 10^4 pfu/ml. Tables 1–3 show the stimulation obtained in individual experiments with groups A–C following challenge by antigen in a concentration giving optimal activation by UV RV in each experiment. In these tables the stimulation is expressed as 1) *specific stimulation* i.e. the difference between thymidine incorporation of cells incubated with virus (L-RV or UV RV) and that of cells incubated with CC, and 2) *lymphocyte stimulation index (LSI)* i.e. the thymidine incorporation of cells grown with

TABLE 3 *Results of Stimulation by UV-inactivated Rubella Virus (UV-RV) and Live Rubella Virus (L-RV) of Lymphocytes from Donors with HI-titres Less than 5 (group C)*

HI-titre	UV-RV		L-RV	
	Specific stimulation a)	LSI b)	Specific stimulation a)	LSI b)
< 5	75	1.4	17	1.1
< 5	71	1.4	63	1.3
< 5	282	1.3	(−822) 0	0.2
< 5	433	1.2	(−331) 0	0.8
< 5	118	1.2	646	2.4
< 5	64	1.2	(−37) 0	0.9
< 5	52	1.1	(−15) 0	1.0
< 5	26	1.1	55	1.[illegible]
Mean ± SD	140 ± 142	1.2	(−56) 0 ± 383	1.1

a) cpm/ml virus — cpm/ml cc

b) $\frac{\text{cpm/ml virus}}{\text{cpm/ml cc}}$

cyte activation is observed following stimulation with L-RV and the mean specific stimulation of this group is significantly lower than that of group B cultures (Table 2) stimulated with L-RV ($p < 0.025$)

DISCUSSION

This paper presents a study of antigen-specific lymphocyte stimulation by rubella virus in which comparison is made of the effects of live and UV-inactivated rubella virus on lymphocytes from donors with different serological immunity against rubella. The virus used was of high purity and the culture medium was supplemented by HI negative homologous serum in order to minimize possible interference between viral antigens and preformed anti-rubella antibodies.

Our results confirm earlier observations (9 16, 17 23) since they show a moderate degree of antigen-specific lymphocyte stimulation after incubation with UV-inactivated rubella virus. We can also confirm that there is no obvious correlation between the degree of lymphocyte stimulation and the magnitude of the HI titres of the lymphocyte donors (16, 17 23). In fact, the average LSI of the groups with high and low HI-titres were virtually the same. However no stimulation occurred when cells from non-immune donors were cultured in the presence of L-RV or UV RV which establishes the immunological specificity of the reaction

Following stimulation by L-RV striking differences were observed between the group with low HI titres and the group with moderate to high titres. In the former the LSI obtained were of the same magnitude as those seen in cultures incubated with UV RV. However in the latter group, the stimulation was nil after incubation with L-RV. The reason for this non-reactivity of lymphocytes from high-titre donors is not clear. The obvious possibility that antibodies neutralize the viral antigens can be ruled out, since such antibodies would neutralize the UV RV as well. Furthermore, the cells were cultured in medium supplemented by antibody-free serum. There is an interesting analogy between this nonreactivity and the inhibition of PHA stimulation by L-RV. In the latter system, no inhibition was seen when the lymphocyte donor lacked serological immunity against rubella, whereas the inhibition was considerable in cases with high titres of anti-rubella antibodies (8). Both phenomena could be explained on the assumption that L-RV in some

TABLE 1 *Results of Stimulation by UV-inactivated Rubella Virus (UV RV) and Live Rubella Virus (L-RV) of Lymphocytes from Donors with HI-titres Ranging from 40 to 160 (group A)*

HI titre	UV RV		L-RV	
	Specific stimulation a)	LSI b)	Specific stimulation a)	LSI b)
160	2101	4 2	(—508) 0	0.2
160	1387	2.3	(—822) 0	0.2
80	1494	6 8	(—54) 0	0 8
80	1414	4 4	66	1.2
40	1224	5 1	1525	6.2
40	394	4 0	131	2.0
40	232	2.1	(—4) 0	1.0
40	41	1 1	158	1 4
40	(—9) 0	1 0	(—34) 0	0.8
Mean ± SD	920 ± 761	3 4	51 ± 612	1.3

a) cpm/ml virus — cpm/ml cc

b) $\frac{\text{cpm/ml virus}}{\text{cpm/ml cc}}$

TABLE 2. *Results of Stimulation by UV-inactivated Rubella Virus (UV RV) and Live Rubella Virus (L-RV) of Lymphocytes from Donors with HI-titres Ranging from 5 to 20 (group B)*

HI titre	UV RV		L-RV	
	Specific stimulation a)	LSI b)	Specific stimulation a)	LSI b)
20	1352	4.5	2088	6.5
20	141	1 6	160	1.6
20	86	1 4	193	2.0
10	1524	3 1	1777	3.4
10	725	2.3	1093	2.9
5	746	6 1	342	3.3
5	1738	2 4	2978	3.3
Mean ± SD	902 ± 657	3 1	1233 ± 1089	3.3

a) cpm/ml virus — cpm/ml cc

b) $\frac{\text{cpm/ml virus}}{\text{cpm/ml cc}}$

ident, the LSI ranging between 1 0 and 6 8 (Tables 1 and 2) Obviously there is no correlation between the LSI and HI titres. The mean specific stimulations following activation by UV RV in both groups are significantly higher than that of group C ($p < 0\ 01$) Incubation with L-RV in the experiments with group B gives a stimulation comparable with that obtained after incubation with UV RV (Table 2) the mean specific stimulation being significantly higher than that of group C cultures challenged with L-RV ($p < 0\ 025$) However in group A (Table 1) with the exception of one experiment, no lympho-

15 *Scriba, M.*, Stimulation of peripheral blood lymphocytes by Herpes simplex virus in vitro. Infection and Immunity *10* 430–436 1974.

16. *Simons M J & Fitzgerald M G.* Rubella virus and human lymphocytes in culture Lancet *ii* 937–940 1968.

17 *Smith K. A., Chess, L. & Mardiney M R. J* The relationship between rubella hemagglutination inhibition antibody (HIA) and rubella induced in vitro lymphocyte tritiated thymidine incorporation. Cell. Immunol. *8* 321 327 1973.

18. *Soontil F J C. J & van der Veen J.*, Evidence for macrophage mediated effect of polio virus on the lymphocyte response to phytohemagglutinin J Immunol. *111* 1411–1419 1973

19 *Vähäkangas P & Vaheri A.* Large-scale purification of rubella virus and preparation of an experimental split rubella virus vaccine. Appl. Microbiol. *22* 255–259 1971

20 *Vaheri, A von Bonsdorff C.-H., Venkari T., Hovi T & Vähäkangas P.*, Purification of rubella virus particles. J gen. Virol. *5* 39–46, 1969

21 *Valle M J., Bobrove A M., Strober S & Merigan T C.*: Immune specific production of interferon by human T-cells in combined macrophage-lymphocyte cultures. J Immunol. *114* 435–441 1975

22. *Venkari, T & Brumerick-Klein E.*, Lymphocyte responses to rubella antigen and phytohemagglutinin after administration of the RA 27/3 strain of live attenuated rubella vaccine. Infection and Immunity *11*: 748–753 1975

23. *Vesikari T., Kanra, G Y., Buimovici-Klein E. & Cooper L. Z.*: Cell-mediated immunity in rubella assayed by cytotoxicity of supernatants from rubella virus-stimulated human lymphocyte cultures. Clin. exp Immunol. *19* 33–43 1975

way renders the lymphocytes anergic. For example, L-RV might interfere with the stimulation process, or it could infect and replicate in the stimulated cells and thus impair the growth and proliferation of these cells. Alternatively L-RV may infect the monocytes (18) and prevent these cells from cooperating with the lymphocytes, which is necessary for *in vitro* stimulation of lymphocytes (4 12 21) However the question as to why such an anergy should be induced only in lymphocytes from high t tre donors requires additional explanation.

Another possibility is that L-RV exerts both stimulating and inhibiting effects on lymphocytes *in vitro* The proliferation induced would thus be a net result of these two effects of the virus L-RV may induce the production of a substance which inhibits the proliferation of stimulated lymphocytes Assuming that the amount of inhibiting substance produced is related to the serological immunity of the lymphocyte donor the differences in lymphocyte activation between high titre and low titre donors could be explained One known growth inhibitor of *in vitro* stimulated lymphocytes is interferon (7) which is synthesized after immune specific lymphocyte stimulation (4 12 21) Other possible inhibitors are complexes between rubella virus and anti rubella antibodies which it has been claimed inhibit PHA stimulation of lymphocytes (6) Hypothetically complexes between rubella virus and antibodies produced *in vitro* could inhibit the proliferation of stimulated lymphocytes in the present system. Whichever mechanism is operative there are indications that the inhibiting effect is in some way connected with the virulence of the virus preparation (14)

The authors wish to thank Mrs. *Ulla Strand* and Miss *Kristina Groth* for skilful technical assistance.

REFERENCES

1 *Cole P J & Molyneux M E.* Lymphocyte reactivity to influenza virus in man Immunol. *29* 749–754 1975

2. *Cooper L. Z., Matters B Rosenblum, J K. & Krugman S.*, Experience with a modified rubella hemagglutination inhibition antibody test JAMA *207* 89–93 1969

3 *Elves M W Roath, S & Israels, M C. G.* The response of lymphocytes to antigen challenge in vitro. Lancet i 806–807 1963.

4 *Epstein L. B., Stevens D A. & Merigan, T C* Selective increase in lymphocyte interferon response to vaccinia antigen after revaccination. Proc. Nat. Acad. Sci. USA 69 2632–2636 1972.

5 *Fabiyi A., Sever J L. Ratner N & Caplan B* Rubella virus Growth characteristics and stability of infectious virus and complement-fixing antigen. Proc. Soc. Exp. Biol. Med. *122* 392–396 1966.

6 *Lee J C & Sigel, M M* A differential effect of IgM and IgG antibodies on the blastogenic response of lymphocytes to rubella virus. Cell. Immunol. *13* 22–31 1974

7 *Lindahl-Magnusson P Leary P & Gresser I* Interferon inhibits DNA synthesis induced in mouse lymphocyte suspensions by phytohaemagglutinin or by allogenic cells. Nature (New Biol.) *237* 120–121 1972.

8 *Møller R & Sörén L.* In vitro effects of rubella virus, strain RA 27/3 on human lymphocytes. I Viral inhibition of mitogen stimulation in relation to rubella haemagglutination inhibition antibodies. Acta path. microbiol. scand. Sect. C, *85* 49–56, 1977

9 *Morag A., Morag, B Bernstein J M., Beutner K. & Ogra P L.* In vitro correlates of cell-mediated immunity in human tonsils after natural or induced rubella virus infection. J Infect. Dis. *131* 409–416 1975

10 *Notkins A L. Mergenhagen S E. & Howard R. J* Effect of virus infections on the function of the immune system. Ann. Rev Microbiol. *24* 525–538, 1970

11 *Olson G B. Dent P B. Rawls W E. South M A Montgomery J R Melnick J L & Good R A* Abnormalities of in vitro lymphocyte responses during rubella virus infections. J Exp. Med. *128* 47–28 1968.

12 *Rasmussen L. E Jordan G W., Stevens, D A & Merigan T C.,* Lymphocyte interferon production and transformation after Herpes simplex infections in humans. J Immunol. *112* 728–735 1974

13 *Rosenberg G L. Farber P A & Notkins, A L.* In vitro stimulation of sensitized lymphocytes by Herpes simplex virus and vaccinia virus. Proc. Nat. Acad. Sci. USA *69* 756–760, 1972.

14 *Savel H & Phillips C A* Decreased inhibition of phytohemagglutinin-induced lymphocyte transformation by attenuated strains of rubella strains. Microbios *5* 49–52, 19 0.

Acta path. microbiol. scand. Sect. C, 85: 317-323, 1977

MODULATION OF HUMAN LEUCOCYTE MIGRATION INHIBITORY FACTOR (LIF) BY 3' 5'-CYCLIC AMP, 3' 5'-CYCLIC GMP AND AGENTS KNOWN TO INFLUENCE INTRACELLULAR CYCLIC NUCLEOTIDE METABOLISM

K. Bendtzen and J. Palit

Laboratory of Clinical Immunology, Medical Department TA,
Rigshospitalet University Hospital, Copenhagen, Denmark

Bendtzen, K. & Palit, J. Modulation of human leucocyte migration inhibitory factor (LIF) by 3' 5'-cyclic AMP, 3' 5'-cyclic GMP and agents known to influence intracellular cyclic nucleotide metabolism. Acta path. microbiol. scand. Sect. C, 85: 317-323, 1977.

A study was made of the effects of 3' 5'-cyclic AMP (3' 5' cAMP), 3' 5'-cyclic GMP (3' 5' cGMP) and their dibutyryl derivatives, and the effects of various both cyclic and non-cyclic nucleotides and of nucleosides on the in vitro migration of human peripheral blood leucocytes under agarose and on the activity of leucocyte migration inhibitory factor (LIF). Leucocyte migration was not significantly influenced by any of the above-mentioned drugs. However LIF activity was significantly depressed by 3' 5' cAMP and dibutyryl 3' 5' cAMP, whereas cells challenged with 10^{-4}M of the other drugs, including 2' 3' cAMP, 3'-AMP, 5' AMP and adenosine, showed unreduced migration inhibition under standard test conditions. A possible role of 3' 5' cAMP in the mechanism of LIF action was supported further by experiments with various drugs known to influence intracellular 3' 5' cAMP metabolism. Treatment of leucocytes with the 3' 5' cAMP generating, beta-adrenergic agent isoproterenol (10^{-4}M) caused a rapid, transient reduction of LIF activity as compared to LIF-treated controls. The alfa-adrenergic agent norepinephrine (10^{-4}M) was ineffective. Treatment of leucocytes with the phosphodiesterase inhibitors papaverine (10^{-4}M) and dipyridamole (2×10^{-4}) enhanced their motility and enabled them to escape migration inhibition as compared to LIF-treated controls. 3' 5' cGMP may also participate in the expression of LIF activity since cells treated with 3' 5' cGMP partially escaped migration inhibition during the first 3 hours of migration.

Key words. Leucocyte migration inhibitory factor (LIF); 3' 5'-cyclic AMP and 3' 5'-cyclic GMP; catecholamines; phosphodiesterase inhibitors.

K. Bendtzen, Laboratory of Clinical Immunology, Medical Department TA, Rigshospitalet University Hospital, Tagensvej 18, DK 2200 Copenhagen N, Denmark.

Discussion

Two populations of Fc receptors have been demonstrated in human placentae. Those on stem vessel endothelium can be manifest both by immunofluorescence with heat aggregated IgG and by haemadsorption with antibody sensitized erythrocytes, whereas Fc receptors on trophoblastic tissue are manifest in haemadsorption reaction and not in immunofluorescence reaction. The lack of demonstrable immunofluorescence reaction with trophoblastic tissue may be explained by postulating widely-spaced Fc receptors of low affinity on trophoblastic tissue, that can be cross-linked by erythrocyte-bound IgG but not by heat-aggregated IgG Previous studies have shown that the receptors manifest in both the haemadsorption and immunofluorescence reactions on full-term placental tissue express a specificity for the Fc region of IgG (5 7) The haemadsorption reaction can be inhibited by native IgG (7) unlike the immunofluorescent reaction which required aggregated or complexed IgG (5)

The two Fc receptor populations in human placenta may have important biological significance. The Fc receptors on trophoblastic tissue may be involved in the active placental transfer of maternal IgG from mother to foetus. The receptor activity on placental endothelium may function to help protect the foetus from immune complexes formed within the placenta following transfer of maternal IgG antibody to paternally-derived alloantigens of the foetus.

There is the further possibility that Hofbauer cells (macrophages) present in the mesenchymal stroma of chorionic villi (2) may also express Fc receptor activity No evidence for this was obtained by immunofluorescence studies using aggregated IgG However very occasional small clusters of indicator erythrocytes could be seen binding in the mesenchymal stroma using the haemadsorption technique. Membrane Fc receptors on cells corresponding morphologically to Hofbauer cells have, nevertheless, been described using antibody-sensitized erythrocytes rosetting techniques on cell suspensions obtained from human placental tissue (9) although further studies may be needed to definitely characterize such rosetting cells (3, 9)

Indeed, the presence of Fc receptor bearing cells in ectodermal, endodermal and, possibly mesodermal tissue in human placentae makes it surprising that a homogenous Fc receptor population has been claimed to be observed in membrane preparations from the villous portions of full term human placentae (8)

This work was supported in part by grants from the *Norwegian Cancer Society* and the *Royal Society* London.

We would like to thank Dr *O Tönder* for helpful advice and discussion.

References 1 *Faulk W P & Johnson P M* Clin. exp Immunol. 27 365–375 1977—2. *Fox, H.* J Path. Bact. 93 710–719 1976.—3 *Jenkinson E. J Billington W D & Elson J.* Clin. exp Immunol. 23 456–461 1976—4 *Johnson P M & Faulk W P.* Clin. exp. Immunol. 24 435–440 1976.—5 *Johnson P M Faulk W P & Wang A -C* Immunology 31 659–664 1976—6 *Johnson P M., Trenchev P & Faulk W P* Clin. exp. Immunol. 22 133–138, 1975.—7 *Matre R., Tönder O & Endresen C* Scand. J Immunol. 4 741–745 1975—8 *McNabb T Koh, T Y Dorrington K J & Painter R H* J Immunol. 117 882–888 1976—9 *Moskalewski S., Ptak W & Czarnik Z* Biol. Neonate. 26 268–273 1975

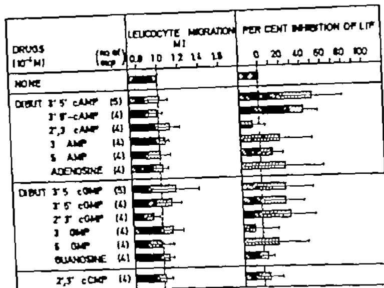


Fig 1 Effects on leucocyte migration and LIF activity of various cyclic and non-cyclic nucleotides and nucleosides. For calculating the migration index (MI) and the inhibition of LIF activity see Materials and Methods.

RESULTS

LIF Modifying Effects of 3' 5' cAMP and 3' 5' cGMP

As documented previously LIF treated leucocytes partially escaped migration inhibition in the presence of dibutyryl 3' 5 cAMP ($\geq 10^{-4}$M) and 3' 5 cAMP ($\geq 10^{-4}$M) whereas dibutyryl 3' 5 cGMP and 3' 5 cGMP ($\leq 10^{-4}$M) was ineffective in modifying LIF activity (8) To test the specificity of these findings, migration inhibition experiments were carried out with leucocytes treated with various nucleotides and nucleosides of the purine bases adenine and guanine. In addition, a cyclic nucleotide of a pyrimidine base was tested

Initially the direct effect of the drugs on leucocyte migration was studied. As shown in Fig 1 neither stimulation nor inhibition of leucocyte motility was observed. When testing the effect of drugs on LIF activity only 3' 5 cAMP and its lipid soluble derivative reduced LIF activity significantly ($P < 0.05$ Mann-Whitney rank sum test) (Fig. 1) Thus, the LIF inhibitory effect of 3' 5 cAMP appears to be highly specific since other cyclic nucleotides including 2' 3' cAMP were ineffective, as were mononucleotides such as 3'AMP and 5 AMP the latter of which is a product of cellular 3' 5 cAMP metabolism.

The stability of drugs (notably 3' 5 cGMP (13)) in cell cultures may be rather limited. In the foregoing experiments, an LIF modifying effect of a drug might therefore have passed unnoticed because of drug inactivation long before termination of the cell cultures. To test this possibility kinetic experiments were carried out, allowing cells to migrate for 22 h with intermediate determination of areas after 3 h and 6 h of migration. As shown in Fig. 2, the time course of leucocyte migration in medium alone (control) was not altered either by dibutyryl 3' 5 cAMP or by dibutyryl 3' 5 cGMP As also shown in Fig 2, the LIF inhibitory effect of dibutyryl 3' 5 cAMP which is readily seen after 22 h of leucocyte migration, can be observed from the very beginning of the culture period. It is of interest that an early LIF inhibitory activity of dibutyryl 3' 5 cGMP is also seen, the effect of which subsides after less than six hours of migration. This pattern of response

Received 28.II.77 Accepted 19.IV.77

Lymphocytes stimulated with antigen or mitogen have been found to produce a number of soluble mediators, lymphokines, which are defined in terms of their biological function *in vitro*. The mechanisms underlying the action of these mediators are largely unknown. However, recent data from this laboratory suggest that 3' 5' cAMP may modulate the leucocyte response to one of these lymphokines—the leucocyte migration inhibitor factor (LIF) (8).

To investigate further the role of 3' 5' cAMP and 3' 5' cGMP in the process of migration inhibition and the possibility that LIF itself might alter the intracellular concentration of cyclic nucleotides, the LIF modifying effects of a variety of drugs known to influence the metabolism of these important second messengers were tested.

MATERIALS AND METHODS

LIF Production

LIF was produced and isolated as described previously (3). Briefly, human peripheral blood lymphocytes were incubated with (LIF rich) and without (control) concanavalin A (con A) for 22 h, the cells were removed by centrifugation, and the control supernatant was reconstituted with con A. Both supernatants were then desalted and lyophilized. Pooled LIF rich and control supernatants were depleted of con A and partially purified by Sephadex G-100 column chromatography. LIF containing fractions (MW 40 000–80 000) and their control counterparts were pooled, lyophilized and stored at 4° C until use.

Assay for LIF

An indirect leucocyte migration agarose technique was employed, using unrelated peripheral blood leucocytes as migrating cells (3). 22 × 10⁶ cells per 90 µl culture supernatant were tested in 7 µl samples for migration under agarose, and the migration index (MI) was determined: *MI = Mean area of cell migration in LIF-rich supernatant / mean area of cell migration in control supernatant.*

Drugs

Adenosine 2' 3' and adenosine 3' 5'-cyclic monophosphoric acid, N⁶ O²'-dibutyryl adenosine 3' 5'-cyclic monophosphoric acid, guanosine 2' 3' and guanosine 3' 5'-cyclic monophosphoric acid, N² O²'-dibutyryl guanosine 3' 5'-cyclic monophosphoric acid, cytidine 2' 3'-cyclic monophosphoric acid, adenosine 3' and adenosine 5'-monophosphate, adenosine, guanosine 3' and guanosine 5' monophosphate, guanosine, DL-isoproterenol sulphate, carbomylcholine chloride and theophylline were purchased from Sigma (St. Louis, Mo., U.S.A.). All nucleotides were obtained as sodium salts. L-norepinephrine-L tartrate and imidazole were from Merck (Darmstadt, West Germany) and dipyridamole was from C. H. Boehringer Sohn (Ingelheim am Rhein, West Germany). All these chemicals were dissolved or diluted in Hanks balanced salt solution and adjusted to pH 7.2 at 37° C before use. L-epinephrine (Merck) was dissolved (10^{-2}M) in distilled water acidified with 0.1 ml 1 N HCl and then adjusted to pH 7.2 with 1 N NaOH. Papaverine (Sigma) was dissolved (10^{-2}M) in 10 per cent v/v dimethyl sulphoxide in distilled water. The final concentration of dimethyl sulphoxide (always less than 0.1 per cent v/v) did not affect leucocyte migration or LIF activity. All drugs were stored in frozen state and fresh solutions were prepared each week.

Effect of Drugs on Leucocyte Migration and on LIF Activity

Lyophilized LIF rich and control supernatants were dissolved in HEPES-buffered culture medium TC-199 with penicillin 100 i.u. and streptomycin 100 µg per ml (Difco Laboratories, Michigan, U.S.A.) (TC-199). Ninety µl three times concentrated LIF-rich and control supernatants were then incubated for 30 min at 37° C, either with a) 10 µl TC-199 as a control for initial lymphokine activity or b) 10 µl of drug solution to give the final concentration indicated in the figures. Leucocytes were then mixed with each supernatant to contain 10 per cent (v/v) horse serum. After further incubation for 60 min at 37° C, the cells were allowed to migrate under agarose for 22 h, unless otherwise stated.

The direct effect of a drug on leucocyte migration was determined as a migration index (MI) calculated as follows: *MI = Mean area of cell migration after drug treatment / mean area of cell migration in medium alone.*

The LIF inhibitory effect of each drug was calculated by comparing the MI of supernatants (LIF rich and its control counterpart) treated with the drug ($MI_{treated}$) with the MI of parallel but untreated supernatants ($MI_{control}$) using the formula: *Per cent inhibition of LIF activity =* $(MI_{treated} - MI_{control}) / 1 - MI_{control}) \times 100$

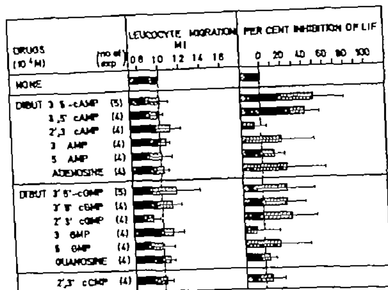


Fig. 1. Effects on leucocyte migration and LIF activity of various cyclic and non-cyclic nucleotides and nucleosides. For calculating the migration index (MI) and the inhibition of LIF activity see Materials and Methods.

RESULTS

LIF Modifying Effects of 3' 5' cAMP and 3' 5' cGMP

As documented previously LIF treated leucocytes partially escaped migration inhibition in the presence of dibutyryl 3' 5' cAMP ($\geq 10^{-4}$M) and 3' 5' cAMP ($\geq 10^{-4}$M) whereas dibutyryl 3' 5' cGMP and 3' 5' cGMP ($\leq 10^{-4}$M) was ineffective in modifying LIF activity (8). To test the specificity of these findings, migration inhibition experiments were carried out with leucocytes treated with various nucleotides and nucleosides of the purine bases adenine and guanine. In addition, a cyclic nucleotide of a pyrimidine base was tested.

Initially the direct effect of the drugs on leucocyte migration was studied. As shown in Fig. 1 neither stimulation nor inhibition of leucocyte motility was observed. When testing the effect of drugs on LIF activity only 3' 5' cAMP and its lipid soluble derivative reduced LIF activity significantly ($P < 0.05$ Mann-Whitney rank sum test) (Fig. 1). Thus, the LIF inhibitory effect of 3' 5' cAMP appears to be highly specific, since other cyclic nucleotides including 2' 3' cAMP were ineffective, as were mononucleotides such as 3'AMP and 5'-AMP, the latter of which is a product of cellular 3' 5' cAMP metabolism.

The stability of drugs (notably 3' 5' cGMP (13)) in cell cultures may be rather limited. In the foregoing experiments, an LIF modifying effect of a drug might therefore have passed unnoticed because of drug inactivation long before termination of the cell cultures. To test this possibility kinetic experiments were carried out, allowing cells to migrate for 22 h with intermediate determination of areas after 3 h and 6 h of migration. As shown in Fig. 2, the time course of leucocyte migration in medium alone (control) was not altered either by dibutyryl 3' 5' cAMP or by dibutyryl 3' 5' cGMP. As also shown in Fig. 2, the LIF inhibitory effect of dibutyryl 3' 5' cAMP, which is readily seen after 22 h of leucocyte migration, can be observed from the very beginning of the culture period. It is of interest that an early LIF inhibitory activity of dibutyryl 3' 5' cGMP is also seen, the effect of which subsides after less than six hours of migration. This pattern of response

Received 28.ii 77 Accepted 19.iv 77

Lymphocytes stimulated with antigen or mitogen have been found to produce a number of soluble mediators lymphokines which are defined in terms of their biological function in vitro The mechanisms underlying the action of these mediators are largely unknown However recent data from this laboratory suggest that 3 5 cAMP may modulate the leucocyte response to one of these lymphokines–the leucocyte migration inhibitor factor (LIF) (8)

To investigate further the role of 3 5 cAMP and 3 5 cGMP in the process of migration inhibition and the possibility that LIF itself might alter the intracellular concentration of cyclic nucleotides the LIF modifying effects of a variety of drugs known to influence the metabolism of these important second messengers were tested

MATERIALS AND METHODS

LIF Production

LIF was produced and isolated as described previously (3) Briefly human peripheral blood lymphocytes were incubated with (LIF-rich) and without (control) concanavalin A (con A) for 22 h the cells were removed by centrifugation, and the control supernatant was reconstituted with con A Both supernatants were then desalted and lyophilized Pooled LIF rich and control supernatants were depleted of con A and partially purified by Sephadex G-100 column chromatography LIF containing fractions (MW 40 000–80 000) and their control counterparts were pooled lyophilized and stored at 4 C until use.

Assay for LIF

An indirect leucocyte migration agarose technique was employed using unrelated peripheral blood leucocytes as migrating cells (3) 22×10^6 cells per 90 µl culture supernatant were tested in 7 µl samples for migration under agarose and the migration index (MI) was determined *MI = Mean area of cell migration in LIF rich supernatant / mean area of cell migration in control supernatant*

Drugs

Adenosine 2 3 and adenosine 3 5 -cyclic monophosphoric acid N 0^2-dibutyryl adenosine 3 5 -cyclic monophosphoric acid guanosine 2' 3' and guanosine 3 5 -cyclic monophosphoric acid N^2 0^2-dibutyryl guanosine 3' 5 -cyclic monophosphoric acid cytidine 2 3'-cyclic monophosphoric acid adenosine 3 and adenosine 5 -monophosphate adenosine guanosine 3' and guanosine 5 monophosphate guanosine DL-isoproterenol sulphate carbomylcholine chloride and theophylline were purchased from Sigma (St. Louis, Mo U.S.A.) All nucleotides were obtained as sodium salts. L-norepinephrine L-tartrate and imidazole were from Merck (Darmstadt, West Germany) and dipyridamole was from C. H Boehringer Sohn (Ingelheim am Rhein West Germany) All these chemicals were dissolved or diluted in Hanks balanced salt solution and adjusted to pH 7.2 at 37 C before use L-epinephrine (Merck) was dissolved (10^{-2}M) in distilled water acidified with 0 1 ml 1 N HCl and then adjusted to pH 7.2 with 1 N NaOH. Papaverine (Sigma) was dissolved (10^{-2}M) in 10 per cent v/v dimethyl sulphoxide in distilled water The final concentration of dimethyl sulphoxide (always less than 0 1 per cent v/v) did not affect leucocyte migration or LIF activity All drugs were stored in frozen state and fresh solutions were prepared each week.

Effect of Drugs on Leucocyte Migration and on LIF Activity

Lyophilized LIF-rich and control supernatants were dissolved in HEPES-buffered culture medium TC-199 with penicillin 100 i.u. and streptomycin 100 µg per ml (Difco Laboratories, Michigan, U.S.A) (TC-199) Ninety µl three times concentrated LIF rich and control supernatants were then incubated for 30 min at 37 C, either with a) 10 µl TC-199 as a control for initial lymphokine activity or b) 10 µl of drug solution to give the final concentration indicated in the figures. Leucocytes were then mixed with each supernatant to contain 10 per cent (v/v) horse serum. After further incubation for 60 min at 37 C, the cells were allowed to migrate under agarose for 22 h, unless otherwise stated.

The direct effect of a drug on leucocyte migration was determined as a migration index (MI) calculated as follows *MI = Mean area of cell migration after drug treatment / mean area of cell migration in medium alone*

The LIF inhibitory effect of each drug was calculated by comparing the MI of supernatants (LIF rich and its control counterpart) treated with the drug ($MI_{treated}$) with the MI of parallel but untreated supernatants ($MI_{control}$) using the formula *Per cent inhibition of LIF activity =* $(MI_{treated} - MI_{control}) / (1 - MI_{control}) \times 100$

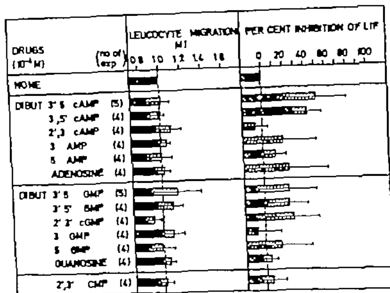


Fig 1 Effects on leucocyte migration and LIF activity of various cyclic and non-cyclic nucleotides and nucleosides For calculating the migration index (MI) and the inhibition of LIF activity see Materials and Methods

RESULTS

LIF Modifying Effects of 3' 5' cAMP and 3' 5' cGMP

As documented previously LIF treated leucocytes partially escaped migration inhibition in the presence of dibutyryl 3' 5' cAMP ($\geq 10^{-4}$ M) and 3' 5' cAMP ($\geq 10^{-3}$ M) whereas dibutyryl 3' 5' cGMP and 3' 5' cGMP ($\leq 10^{-3}$ M) was ineffective in modifying LIF activity (8) To test the specificity of these findings, migration inhibition experiments were carried out with leucocytes treated with various nucleotides and nucleosides of the purine bases adenine and guanine. In addition, a cyclic nucleotide of a pyrimidine base was tested.

Initially the direct effect of the drugs on leucocyte migration was studied. As shown in Fig 1 neither stimulation nor inhibition of leucocyte motility was observed. When testing the effect of drugs on LIF activity only 3' 5' cAMP and its lipid soluble derivative reduced LIF activity significantly ($P < 0.05$ Mann-Whitney rank sum test) (Fig 1) Thus, the LIF inhibitory effect of 3' 5' cAMP appears to be highly specific, since other cyclic nucleotides including 2' 3' cAMP were ineffective, as were mononucleotides such as 3'AMP and 5' AMP the latter of which is a product of cellular 3' 5' cAMP metabolism.

The stability of drugs (notably 3' 5' cGMP (13)) in cell cultures may be rather limited. In the foregoing experiments, an LIF modifying effect of a drug might therefore have passed unnoticed because of drug inactivation long before termination of the cell cultures. To test this possibility kinetic experiments were carried out, allowing cells to migrate for 22 h with intermediate determination of areas after 3 h and 6 h of migration. As shown in Fig 2, the time course of leucocyte migration in medium alone (control) was not altered either by dibutyryl 3' 5' cAMP or by dibutyryl 3' 5' cGMP As also shown in Fig 2, the LIF inhibitory effect of dibutyryl 3' 5' cAMP which is readily seen after 22 h of leucocyte migration, can be observed from the very beginning of the culture period. It is of interest that an early LIF inhibitory activity of dibutyryl 3' 5' cGMP is also seen, the effect of which subsides after less than six hours of migration. This pattern of response

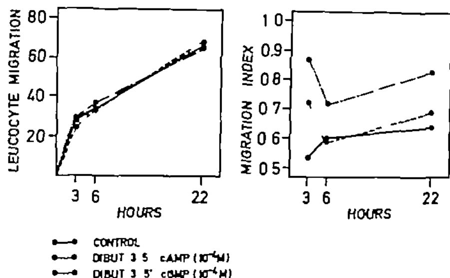


Fig 2 Time course of the effects of dibutyryl 3 5 cAMP and dibutyryl 3 5 cGMP on leucocyte migration (left) and on LIF activity (right) The figure is a typical representative of four different experiments.

DRUGS	(no of exp)	LEUCOCYTE MIGRATION MI (0 8 1.0 1 2 1.4 1 6)	PER CENT INHIBITION OF LIF (0 20 40 60 80 100)
NONE			
10^{-4}M EPINEPHRINE	(5)		
10^{-4}M NOREPINEPHRINE	(5)		
10^{-4}M ISOPROTERENOL	(5)		
10^{-4}M CARBAMYLCHOLINE	(5)		
10^{-2}M IMIDAZOLE	(5)		
10^{-3}M THEOPHYLLINE	(5)		
10^{-3}M PAPAVERINE	(5)		
10^{-4}M PAPAVERINE	(5)		
2×10^{-5}M DIPYRIDAMOLE	(4)		
2×10^{-5}M DIPYRIDAMOLE	(5)		

Fig 3 Effects on leucocyte migration and LIF activity of drugs known to influence intracellular cyclic nucleotide metabolism.

was consistently observed in four different experiments.

LIF Modifying Effects of Drugs Known to Influence Intracellular Levels of 3' 5 cAMP and 3' 5' cGMP

If an LIF induced change in the intracellular concentration of 3 5 cAMP (and of 3 5 cGMP?) is a necessary prerequisite for the expression of LIF activity then drugs known to influence cyclic nucleotide metabolism should be able to modify LIF activity

As shown in Fig 3 both leucocyte migration and LIF activity were unaffected by treatment with three different catecholamines, and also by treatment with a cholinergic stimulator However in four kinetic experiments, one of which is presented in Fig. 4 an early isoproterenol-induced inhibition of LIF activity was observed. This transient effect, induced by a beta-adrenergic stimulator could not be demonstrated after alfa adrenergic stimulation with norepinephrine (Fig 4)

Imidazole at high concentrations is most

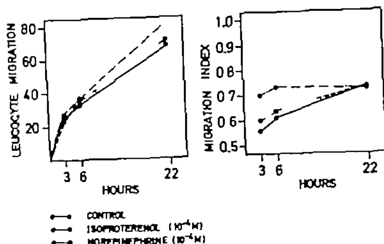


Fig. 4. Time course of the effects of isoproterenol and norepinephrine on leucocyte migration (left) and on LIF activity (right). The figure is typical representative of four different experiments.

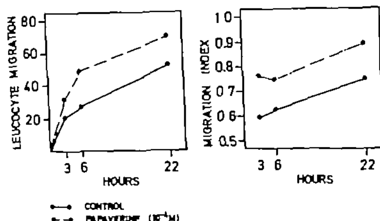


Fig. 5. Time course of the effects of papaverine on leucocyte migration (left) and on LIF activity (right). The figure is typical representative of four different experiments.

frequently quoted as being capable of depressing the levels of 3' 5' cAMP by stimulation of phosphodiesterase (PDE) which catalyzes the hydrolysis of the 3'-phosphoester bond in cyclic nucleotides. Rather than potentiating LIF activity 10^{-4}M imidazole seemed quite unexpectedly to reduce LIF activity although the pooled data of five different experiments (shown in Fig. 3) are not statistically significant.

Finally three different PDE inhibitors were tested. As shown in Fig. 3 leucocyte motility tended to increase after challenge with all three drugs. Moreover papaverine (10^{-4}M) and dipyridamole, even at the lowest concentration tested (2×10^{-6}M) reduced LIF activity markedly ($P < 0.05$ Mann-Whitney rank sum test). Both of these effects of 10^{-4}M papaverine were readily demonstrable at the beginning of cell culture, and the drug induced modifications persisted throughout the entire migration period (Fig. 5).

DISCUSSION

The present results and those of a previous brief report (8) demonstrate that increased levels of 3' 5' cAMP counteract the effect of LIF on human leucocytes. The specificity of this reaction was confirmed by the inability of various other both cyclic and non-cyclic nucleotides to modulate LIF activity. In these experiments, LIF treated leucocytes were challenged with fairly high concentrations of the different drugs throughout the entire migration period. Thus, even a slight effect on LIF should have been detected easily. The LIF inhibitory effect of increased concentrations of 3' 5' cAMP was supported by experiments with papaverine and dipyridamole. These chemically different drugs, capable of raising the intracellular levels of 3' 5' cAMP through their inhibition of PDE (1), markedly reduced LIF activity. These drugs not only prevented migration inhibition but also increased the migration of control cells. This effect was not observed in experiments using exogenous 3' 5' cAMP and may therefore not be due to elevation of 3' 5' cAMP but to some other function of these drugs. However, using a capillary tube technique, *Lomnitzer et al.* (11) reported migration enhancement of dibutyryl 3' 5' cAMP treated control leucocytes. This agent also reduced cell adhesion, which is probably more important for migration inhibition in the capillary tube technique than in the agarose technique (2).

Carbamylcholine, a cholinergic agent capable of elevating 3' 5' cGMP levels in some, but not all, systems (9) did not modify LIF activity. Similar, the beta adrenergic agents isoproterenol and epinephrine, which are well known activators of the 3' 5' cAMP generating enzyme adenyl cyclase, did not reduce LIF activity under standard test conditions. However, kinetic experiments revealed a rapid, transient LIF inhibitory effect of isoproterenol but no effect of the alfa adrenergic agent norepinephrine. Since epinephrine-induced increases in 3' 5' cAMP levels of leucocytes (12) and of macrophages (10) are manifested almost immediately with levels that return to the basic value within a few minutes, these results further seem to indicate a role of 3' 5' cAMP in the process of leucocyte migration inhibition.

In the light of these experiments, a direct involvement, if any, of 3' 5' cAMP with the mechanism of LIF action is probably a long lasting decrease in the intracellular concentration of 3' 5' cAMP. This conclusion is in agreement with experiments showing unreduced migration inhibition of leucocytes pulse treated for 30 min at 37° C with 3' 5' cAMP elevating drugs before challenge with LIF (7).

An additional role of 3' 5' cGMP as modulator of LIF induced migration inhibition cannot be excluded. Thus, a rapid, transient LIF inhibitory effect of exogenous dibutyryl 3' 5' cGMP was observed repeatedly in spite of the reported instability in culture of this nucleotide (13), the LIF inhibitory effect of imidazole may be due to increased levels of 3' 5' cGMP since in some systems imidazole causes inhibition of 3' 5' cGMP PDE rather than activation of 3' 5' cAMP PDE (9), the PDE inhibitors employed in this study may also act as 3' 5' cGMP elevating agents and, most important, 3' 5' cGMP selectively protects inactivation of the LIF molecule by the serine esterase inhibitor phenylmethyl sulphonylfluoride, thus indicating the role of 3' 5' cGMP as a modulator of LIF esterase activity (4, 5, 6, and *Bendtzen*, in preparation).

The study was supported by the *Danish Medical Research Council* and the *P. Carl Petersen Foundation*. The technical assistance of Mrs. *Lady Broe* ... is gratefully acknowledged.

REFERENCES

1. *Amer M. S. & Kreighbaum W. E.*: Cyclic nucleotide phosphodiesterases: Properties, activators, inhibitors, structure-activity relationships, and possible role in drug development. J. Pharm. Sci. 64, 1–37, 1975.
2. *Ax W. & Tautz C.*: Assay of leucocyte migration inhibition under agarose. Behring Inst. Mitt. 54, 77–80, 1974.
3. *Bendtzen K.*: Inhibition of human leukocyte

migration inhibitory factor (LIF) by alfa-L fucose. Acta Allergol. 30 327–338, 1975
4. *Bendtzen, K.* Some physicochemical properties of human leucocyte migration inhibitory factor (LIF) Acta path. microbiol. scand. Sect. C, 84 471–476, 1976.
5. *Bendtzen, K.* Human leukocyte migration inhibitory factor (LIF) I. Effect of synthetic and naturally occuring protease inhibitors. Scand J Immunol. 6 125–131 1977
6. *Bendtzen, K.* Human leukocyte migration inhibitory factor (LIF) II Partial biochemical characterization of the substrate specificities for this lymphokine Scand. J Immunol 6 133–140 1977
7 *Bendtzen K.* Unpublished results.
8. *Bendtzen K & Pelt J I vitro* modulation of human leucocyte migration and migration inhibitory factor (LIF) activity by cyclic 3' 5'-AMP and cyclic 3' 5'-GMP Acta path. microbiol. scand. Sect. C, 85 78–80 1977
9 *Goldberg, N D O'Dea R. F & Haddox M K.* Cyclic GMP In *Greengard P & Robison G A.* (Eds.) Advances in cyclic nucleotide research 5 Raven Press, New York 1975 p. 155.
10. *Higgins, T J & David J R* Effect of isoproterenol and aminophylline on cyclic AMP levels of guinea pig macrophages. Cell. Immunol. 27 1–10 1976.
11 *Lemmitser R., R bsen A R. & Koornhof H J* The effects of cyclic AMP on leucocyte inhibitory factor (LIF) production and on the inhibition of leucocyte migration. Clin. exp. Immunol. 24 42–48 1976.
12. *Ri kin, I Rosenblatt J & Be ker E. L.* The role of cyclic AMP in the chemotactic responsiveness and spontaneous motility of rabbit peritoneal neutrophils. J Immunol. 115 1126–1134 1975
13 *Watson J.* The influence of intracellular levels of cyclic nucleotides on cell proliferation and the induction of antibody synthesis. J exp. Med. 141 97–111 1975.

Acta path. microbiol scand Sect. C 85 324-332, 1977

GEL-PRECIPITATING MEASLES VIRUS ANTIBODIES IN SUBACUTE SCLEROSING PANENCEPHALITIS RELATION TO OLIGOCLONAL IgG PROTEINS IN THE CEREBROSPINAL FLUID AND SERUM, AND THE OCCURRENCE OF TWO SEPARATE FRACTIONS OF ANTIBODY TO VIRUS NUCLEOCAPSIDS

BODVAR VANDVIK

Institute of Immunology and Rheumatology Rikshospitalet and
Department of Neurology Ullevål Sykehus, University of Oslo, Norway

Vandvik, B. Gel-precipitating *measles virus antibodies in subacute sclerosing panencephalitis* Relation to oligoclonal IgG proteins in the cerebrospinal fluid and serum, and the occurrence *of two separate fractions of antibody to virus nucleocapsids. Acta path. microbiol. scand. Sect. C, 85* 324-332, 1977

Two to five separate gel precipitating (GP) antibody activities to sonicated measles virus cell pack antigen were detected by double immunodiffusion and immunoelectrophoresis of sera and cerebrospinal fluids (CSF) from patients with subacute sclerosing panencephalitis (SSPE) The major GP activity was identified as antibody to measles virus nucleocapsids, and a weaker activity as antibody to the virus haemagglutinin. The specificities of the other activities were not determined. Evidence is presented that measles GP antibody activities of both serum and CSF from patients with SSPE are carried by IgG proteins of restricted heterogeneity Two separate and differently charged homogeneous IgG proteins with antibody activities to partially different measles nucleocapsid antigens were detected in about half the patients. The combined use of agarose electrophoresis and measles GP antibody immunoelectrophoresis of serum and CSF proved helpful in establishing rapidly the specific diagnosis of SSPE.

Key words Subacute sclerosing panencephalitis antibodies to measles virus oligoclonal IgG

B Vandvik, Department of Neurology Ullevål Sykehus, University of Oslo, Norway

Received 24.xii.76 Accepted 12.iv 77

Subacute sclerosing panencephalitis (SSPE) is a disease of children and adolescents associated with a measles virus infection of the brain (1, 3 9) Hallmarks of this condition are the occurrence of oligoclonal IgG in the cerebrospinal fluid (CSF) and in most sera (4 5 14) and elevated levels of measles antibodies in serum and CSF (1 10) The oligoclonal IgG of the CSF has been iden

tified as measles virus-specific antibody (17 18) and an association between oligoclonal IgG occurring in serum and measles-specific antibody activities has been demonstrated (12, 18)

The usefulness of immunodiffusion methods for the demonstration of elevated levels and separate populations of measles gel-precipitating (GP) antibodies in material from patients with SSPE has been pointed out previously (2, 8 13) The present paper describes the combined use of protein electrophoresis, double immunodiffusion, and immunoelectrophoresis for the characterization of measles GP antibodies of serum and CSF samples from patients with SSPE. Evidence is presented that separate measles GP antibody activities of both serum and CSF are associated with oligoclonal IgG proteins.

MATERIALS AND METHODS

Sera and CSF

Paired samples of serum and lumbar CSF from 15 patients with SSPE were referred for routine laboratory examination between 1970 and 1975 The diagnosis was established by clinical and electroencephalographic criteria (19) present either when the samples were obtained or t later stages of the disease, the occurrence of oligoclonal IgG in the CSF and the demonstration of reduced serum/CSF ratios (10) of measles irus haemagglutination-inhibiting (HI) and/or complement fixing (CF) antibodies

Paired samples of serum and CSF from 10 patients with non-infectious/non-demyelinating disease 4 patients with defined (one case each of measles encephalitis, herpes simplex encephalitis, mumps meningitis, aricella-zoster myelitis) and 6 patients with presumed ral infection of the CNS and 10 patients with definite (11) MS were studied for comparison. A normal CSF protein pattern was demonstrated in the patients with non-infectious/non-demyelinating disease transudath pattern (5) in patient with acute measles encephalitis and an oligoclonal IgG pattern in the remaining 9 patients with infectious CNS disease and in all the 10 MS patients.

Antisera

Rabbit antisera to human serum, IgG IgA and IgM were raised in this laboratory Rabbit antisera to immunoglobulin κ and λ light chain determinants were purchased from Dakopatts, Denmark. Rabbit antisera to measles virus haemagglutinin (HA) and nucleocapsids (NC) were kindly provided by Dr *E. Norrby* Stockholm.

Virus Antigens

Measles virus cell pack antigen (CPA) was prepared from Edmonston strain A measles virus infected Vero cells (kindly provided by Dr *M Degré* Institute of Microbiology Rikshospitalet, Oslo) essentially as described by *Paushus et al.* (8) A 30 per cent (/v) suspension of packed cells in phosphate buffered saline (PBS) was freeze-thawed five times, sonicated at 20 kH for total of 50–60 s with ice bath cooling, and centrifuged at 1000 × *G* for 5 min. The supernate was used as standard antigen. It was stored at — 20 C and re-sonicated for 10 s before use. Control antigen was prepared from non-infected Vero cell cultures in the same way

A suspension of measles extracellular virus (EV) antigen was prepared from measles virus infected culture supernates concentrated 100 times by vacuum dialysis.

Purified measles irus NC antigen was kindly provided by Dr *E. Norrby* Stockholm (see ref 6)

Agarose and Buffer

Two types of agarose (Indubiose brands A-37 and A-45) were obtained from Industrie Biologique Française, France. Barbital buffer pH 8.6 and ionic strength 0 05 was made from buffer salt purchased from LKB, Sweden.

Double Immunodiffusion

Glass plates were covered with a 1.5 mm layer of 1 per cent A-37 type of agarose in the barbital buffer Opposing wells 2 mm in diameter and 3–4 mm apart were filled with 5 μl/samples of antigen and antibody The plates were left to immunoprecipitate for 18–36 h t room temperature, after which they were washed in saline, rinsed in tap water air dried, and stained with Coomassie Brilliant Blue.

Immunoelectrophoresis

2 × 100 × 200 mm glass plates were covered with 1.5 mm layer of 1.2 per cent agarose (2 parts A-37 and 1 part A-45) in the barbital buffer 5-μl samples were applied in circular wells 2 mm in diameter Electrophoresis was carried out with the same buffer at 15 v/cm for 45 min in water cooled apparatus. Antisera (50–100 μl) or virus antigen (50 μl) were filled into 2 mm wide longitudinal troughs at distance of 3–4 mm from the wells. The plates were left to immunoprecipitate for 18–24 h at room temperature after which they were washed, rinsed, air dried, and stained with Coomassie Brilliant Blue.

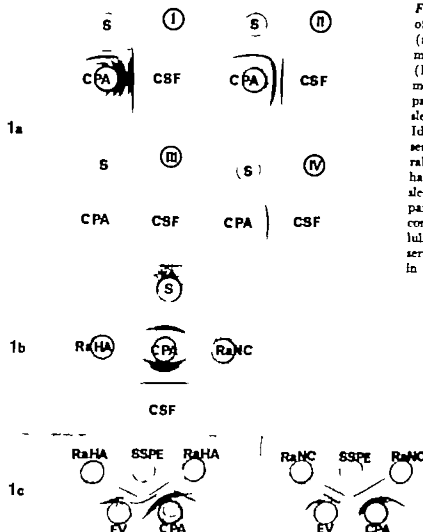


Fig 1 a) Double Immunodiffusion of serum (S) and concentrated CSF (adjusted to IgG between 4 and 6 mg/ml) from two patients with SSPE (I and II) one patient with acute measles encephalitis (III) and one patient with MS (IV) against measles cell pack antigen (CPA) —b) Identification of antibody activities in serum from a patient with SSPE with rabbit antisera specific for measles haemagglutinin (RaHa) and for measles nucleocapsids (RaNC) —c) Comparison between measles CPA and a concentrated suspension of extracellular virus (EV) using the SSPE serum and the rabbit antisera shown in b)

Agarose Electrophoresis

This was carried out in gel plates, under conditions identical to those used for immunoelectrophoresis, but with the 5-µl samples applied in 9 × 0 6 mm premoulded slits, as described elsewhere (17) The plates were stained with Amido black.

Crossed Immunoelectrophoresis

Characterization of immunoglobulins by this method was carried out as described elsewhere (14 17) The first dimension separation was performed as in agarose electrophoresis but with the time of electrophoresis prolonged to 1?0 min. The second dimension immunoelectrophoresis was carried out at 2–3 v/cm for 18–24 h in antibody-containing agarose gel. The plates were stained with Coomassie Brilliant Blue.

RESULTS

Measles GP Antibodies Detected by Double Immunodiffusion

Two to five separate precipitates against the standard (30 per cent v/v) measles CPA were detected in the CSF and sera from the 15 SSPE patients (Fig 1 a)

The major activity of each sample was identified as antibody to measles nucleocapsids (NC) by a reaction of identity with a measles NC-specific rabbit antiserum (Fig 1 b) In many instances the precipitate produced by this antibody was composed of two separate lines

A second and weaker activity occurring in some of the CSF and serum samples was

identified as antibody to measles HA, by a reaction of identity with a measles HA specific rabbit antiserum (Fig. 1 c) The specificities of the remaining one to three activities seen in many of the samples were not determined further

A concentrated suspension of measles EV antigen contained low amounts of the NC and HA antigens and relatively more of a third component present also in the CPA preparation (Fig. 1 c) The nature of this component was not investigated further

No measles GP antibody activity was detected in serum or CSF from ten patients without infectious or demyelinating nervous disease when tested against the standard cell pack antigen. Measles NC antibody was detected in the serum and CSF from a case of acute measles encephalitis and in samples from four out of the ten patients with MS (Fig 1 a) In each of the MS cases, the antibody activity of the CSF was more pronounced than that of the serum.

No measles GP antibody activity was detected in serum of CSF from the nine patients with non-measles infectious disease of the CNS, all of whom had an oligoclonal IgG pattern of the CSF

No SSPE and control sera or CSF samples reacted with the control cell pack antigen prepared from non infected cells.

Measles GP Antibodies Characterized by Immunoelectrophoresis

An electrophoretically uneven distribution of separate measles GP antibody activities was seen in the CSF from all 15 patients with SSPE and in 13 of the matching sera. The precipitate arc morphology varied from one case to another but certain common features were noted

a A major precipitate arc extending through the intermediate and cathodal IgG fraction was produced by the CSF from all 15 SSPE patients. Comparison with the double immunodiffusion GP antibody patterns in each case suggested that this arc was produced by measles NC antibody

The cathodal portion of major arc showed in each case a localized deflection towards the virus antigen through, thus indicating an accumulation of cathodal antibody within a restricted mobility zone. In nine cases a second deflection of the arc occurred in its anodal portion. In seven of these, the fast deflection of the arc spurred with the main arc (Fig. 2 I II)

Selected samples were examined in immunoelectrophoresis with purified measles NC as antigen. In each case, the major precipitate arc was reproduced and appeared to be identical with that developed against CPA. Apparently both the cathodal and the anodal deflections of the arc represented measles NC antibody

b The CSF from all of the 15 SSPE patients contained one to three additional activities producing weaker and, in most cases, less well-defined precipitates in the intermediate and cathodal IgG fraction (Fig 2, I, II) Close scrutiny indicated that in many instances these precipitates were composed of partly spurring and partly crossing arcs occurring within separate and sharply restricted mobility zones.

c. One of more measles GP antibody activities were detected in the patients sera, but these were always weaker and usually fewer than in the matching concentrated CSF samples (Fig. 2 I II) Alle sera gave a measles NC antibody-specific precipitate, but detection of additional precipitates was less constant. Localized deflections of the NC antibody arcs towards the virus antigen trough were seen also in the sera, but were less pronounced that in the CSF

d The electrophoretic mobility of the measles GP antibody activities indicated an association with IgG The CSF of the SSPE patients contained no IgM or only traces of it, in agreement with the intactness of the blood-brain-barrier (BBB) indicated by the CSF electrophoresis in these patients. Probably IgM was not involved in the production of visible precipitate arcs.

Little or no GP antibody was detected in mobility fractions containing IgA. Since no

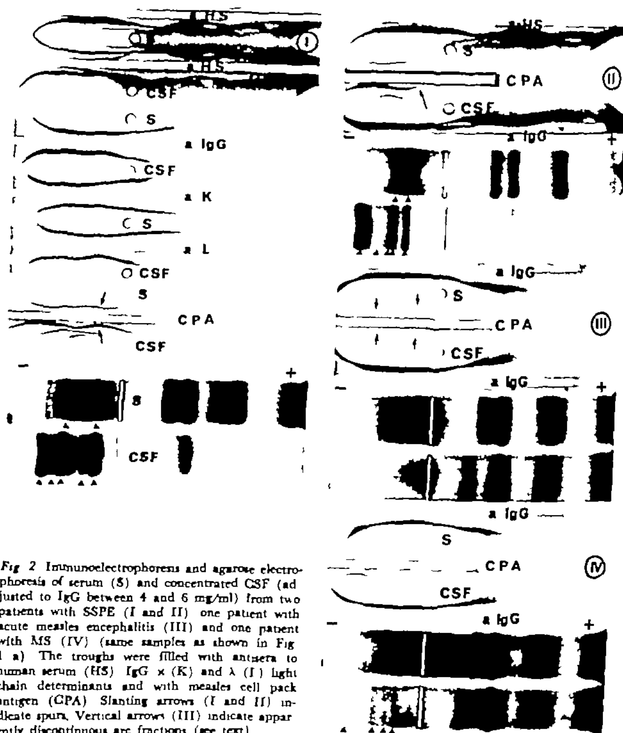


Fig 2 Immunoelectrophoresis and agarose electrophoresis of serum (S) and concentrated CSF (adjusted to IgG between 4 and 6 mg/ml) from two patients with SSPE (I and II) one patient with acute measles encephalitis (III) and one patient with MS (IV) (same samples as shown in Fig 1 a) The troughs were filled with antisera to human serum (HS) IgG κ (K) and λ (L) light chain determinants and with measles cell pack antigen (CPA) Slanting arrows (I and II) indicate spurs. Vertical arrows (III) indicate apparently discontinuous arc fractions (see text)

additional CP activities consistent with IgM or IgA antibodies were detected in the sera the measles CP antibodies were probably carried mainly if not exclusively by IgG proteins.

Immunoelectrophoresis of serum and CSF from the control patients showed a measles *NC antibody arc in samples from* the patients with acute measles encephalitis and from four of the MS patients. The NC antibody arc of serum and CSF in the measles encephalitis case extended through the intermediate and cathodal IgG fraction and showed no localized deflection towards the antigen trough

The serum and CSF arcs were similar in strength, but their appearance suggested that both IgG and IgM antibodies were involved in their formation (Fig 2 III) In the four MS cases the antibody arcs in the CSF were more pronounced than in serum or were visible in the CSF alone, and occurred within electrophoretically restricted zones in the intermediate or cathodal IgG fraction (Fig 2 IV)

Relationship Between Oligoclonal IgG and Measles GP Antibody

Five to eight separate bands of IgG were detected in the CSF of the SSPE patients by agarose electrophoresis (Fig 2 I, II) An association between some of these bands and separate measles GP antibody activities was suggested by similarities in their electrophoretic mobilities. This was particularly the case with major cathodal bands of IgG and the cathodal measles NC antibody fraction. In some samples where bands of IgG were spaced well apart, an association between faster bands of IgG and the anodal measles NP antibody fraction or other GP antibody activities was suggested by the same criteria (Fig 2 I) Serum and CSF samples from six patients were studied sequentially over periods from 3 months to 4 years. Changing patterns of measles GP antibodies in the CSF were observed during periods of disease progression or remission in four of these patients. The same CSF samples showed changes of patterns of oligoclonal IgG (14)

The morphology of the measles GP antibody precipitates of the sera from the SSPE patients suggested the presence of antibodies of restricted heterogeneity in these materials also. In many instances, the GP activities corresponded to faint bands of IgG detected by agarose electrophoresis of the sera. In a number of cases, however apparently restricted GP antibody activities of the sera occurred in mobility fractions which contained no detectable bands of IgG and where the distribution of IgG seemed to be polyclonal (Fig. 2 I)

Isolation of Two Separate Fractions of Measles NC Antibody

As described above, a cathodal and an additional anodal fraction of measles NC antibody occurred in CSF samples from about half the SSPE patients and in some of the matching sera. In order to examine these proteins further measles antibodies were isolated from two sera which contained both fractions of NC antibody

In both cases the antibody eluates obtained by acid elution showed bands or zones of IgG (Fig 3 a) They corresponded to bands of IgG present in the matching CSF as described in more detail elsewhere (18) Separate measles GP antibody activities of the sera were recovered in the antibody eluates and were associated with the homogeneous IgG proteins. In particular the results showed that the sera contained homogeneous IgG proteins with measles GP activity in mobility fractions where bands of IgG could not be demonstrated by the serum electrophoresis (Fig. 3 a)

The cathodal measles NC antibody fraction was eluted mainly at pH 3 and the anodal fraction mainly at pH 2, in both the cases studied. The separate NC antibody fractions were clearly associated with bands of IgG with corresponding mobilities. This was shown by the fact that these bands of IgG reacted with both CPA and measles NC antigen, while other and weaker bands of the same eluates reacted with CPA only (Fig 3 a–c)

The spur formation between the cathodal and the anodal NC antibody fraction was reproduced when the pH 3 and the pH 2 eluates were recombined and examined by immunoelectrophoresis.

Analysis of the light chain determinants of the bands of IgG in the antibody eluates (Fig. 3 b) showed that the anodal fraction of measles NC antibody was in both cases associated with IgG of predominantly λ type. The cathodal fraction was in one case associated with IgG of κ type and in the other with IgG of mainly λ type.

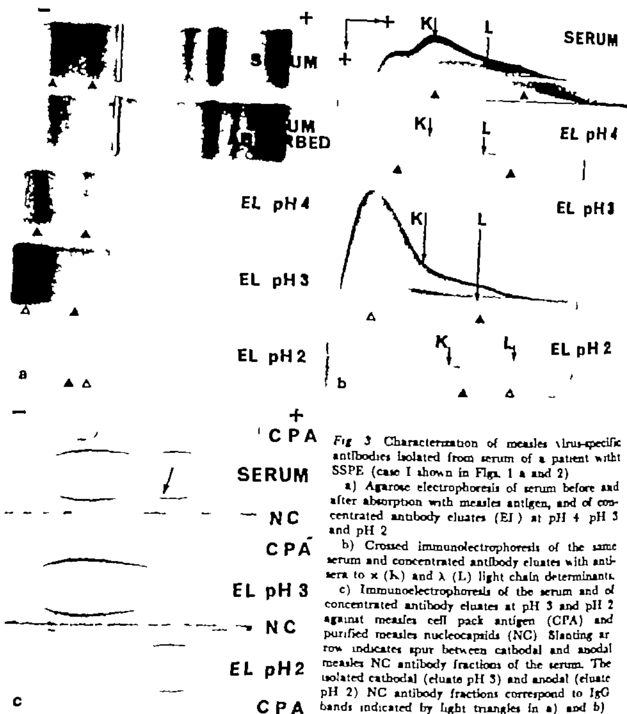


Fig 3 Characterization of measles virus-specific antibodies isolated from serum of a patient with SSPE (case I shown in Figs. 1 a and 2)

a) Agarose electrophoresis of serum before and after absorption with measles antigen, and of concentrated antibody eluates (EL) at pH 4 pH 3 and pH 2

b) Crossed immunoelectrophoresis of the same serum and concentrated antibody eluates with antisera to κ (K) and λ (L) light chain determinants.

c) Immunoelectrophoresis of the serum and of concentrated antibody eluates at pH 3 and pH 2 against measles cell pack antigen (CPA) and purified measles nucleocapsids (NC) Slanting arrow indicates spur between cathodal and anodal measles NC antibody fractions of the serum. The isolated cathodal (eluate pH 3) and anodal (eluate pH 2) NC antibody fractions correspond to IgG bands indicated by light triangles in a) and b)

DISCUSSION

The combination of protein characterization by agarose electrophoresis and antibody identification by immunoelectrophoresis proved useful in obtaining a rapid and specific diagnosis of SSPE. The demonstration of oligoclonal IgG in the CSF associated with an electrophoretically uneven distribution of measles virus GP antibodies, and of a higher antibody activity per unit concentration of IgG in the CSF that in the serum, is direct evidence of the measles hyperimmunization which takes place within the CNS in this disease.

By analogy with its use in SSPE, antibody immunoelectrophoresis to a panel of viral

antigens might prove helpful in determining a local antibody response in patients with other CNS infections and a concomitant occurrence of oligoclonal IgG in the CSF

It should be stressed that a high concentration of measles CPA was used in the present work This permits the demonstration of multiple measles GP antibody activities in SSPE, combined with a low number of GP antibody-positives among other neurological patients. A dilution of the antigen preparation increases the number of NC antibody-positive samples from patients with MS and other diseases (15) but at the cost of demonstrating other GP antibodies.

The occurrence of electrophoretically restricted measles NC antibodies was a prominent feature in the CSF from all SSPE patients studied. However this is not seen exclusively in SSPE, as shown by the evidence of electrophoretically restricted NC antibodies in the CSF from four out of ten patients with MS. These observations are in agreement with other reports (7 10 15 18) indicating that a local synthesis of such antibodies takes place in some patients with MS. The NC antibody activities observed in the CSF of the MS patients were not as pronounced as those observed in the SSPE patients. Furthermore, no additional GP antibodies were detected in the material from the MS patients

The presence of oligoclonal antibodies in the CSF is probably an early feature in SSPE. One of the present patients was examined by brain biopsy shortly after onset of symptoms The biopsy did not reveal histopathological abnormalities specific for SSPE and measles antigen could not be detected by immunofluorescent examination, while the CSF IgG and GP antibody patterns preceded the development of electroencephalographic abnormalities considered to be characteristic for SSPE

An association between separate fractions of oligoclonal IgG in the CSF or serum and antibody activities to different components of measles virus has been described previously in patients with SSPE (7 12, 13 17 18) A tendency for antibodies to measles NC and haemolysin (HL) to be associated with fractions of IgG with more cathodal mobility than antibodies to the virus HA was observed in these patients (7 17) The present finding of a major cathodal NC antibody fraction in all patients is in agreement with these observations. The results show however that a second fraction of measles NC antibody with more anodal mobility occurs in about half the patients.

The frequent occurrence of two separate fractions of measles NC antibody with different mobility suggests some mechanism leading to the selection of cell clones which produce antibodies with certain "preferred" charge characteristics. The significance of this is not clear The spur formations between the cathodal and the anodal NC antibody fractions observed in seven out of fifteen patients indicate that they represent antibodies to partly different antigens of measles NC.

The results of antibody isolation experiments with two SSPE sera show conclusively that the two NC antibody fractions are associated with IgG proteins of restricted heterogeneity both with regard to charge and to light chain determinants. In both cases, the cathodal and the anodal NC antibody proteins were eluted at different levels of pH. One might wonder whether this is associated in some way with differences between the respective NC antigens, e. g. in their susceptibility to become denatured by acids. However the factors determining the level of pH at which a given antibody is detached from its antigen in the method used are not clear and may also be related to the affinities of the antibodies (18)

The antibody specificities of other GP activities migrating with the intermediate and cathodal IgG fraction in immunoelectrophoresis were not determined Some of them possibly represent measles HA antibody since one activity was identified as HA antibody by the double immunodiffusion experiments. Whether the other activities represent antibodies to measles HL or other structural or non-structural components of the virus cannot

be stated. The clarification of this question might shed light on virus or virus-related substances which appear to be immunogenic in the measles infection of the brain in patients with SSPE, but not in other conditions of measles immunization.

Grateful thanks are extended to Mrs. *Helle Dunker* and to Miss *Ruth Nilsen* for their excellent technical assistance. The support from *the Norwegian Research Council for Science and the Humanities*, the *Oslo Multiple Sclerosis Society* and the *Norwegian Odd Fellow Order* is gratefully acknowledged.

REFERENCES

1. *Connolly J H Allen I V Hurwitz L J & Miller J H D* Measles virus antibody and antigen in subacute sclerosing panencephalitis. Lancet *1* 542–544 1967
2. *Dietzman D E Horta-Barbosa L Krebs H Madden D L Fucillo D A & Sever J L* Diagnosis of subacute sclerosing panencephalitis by a simple spinal fluid gel precipitation test for measles. Pediatrics *49* 133–136 1972
3. *Horta-Barbosa, L Fucillo D A Sever J L & Zeman W* Subacute sclerosing panencephalitis Isolation of measles virus from a brain biopsy Nature *221* 974 1969
4. *Karcher D van Sande M & Lowenthal A* Microelectrophoresis in agar gel of proteins of the cerebrospinal fluid and central nervous system. J Neurochem *4* 135–140 1959
5. *Laterre E C* Les protéines du liquide céphalorachidien a l'état normal et pathologique. Arscia, Bruxelles, 1965
6. *Norrby E & Hammarskjöld B* Structural components of measles virus. Microbios *5* 17–29 1972.
7. *Norrby E & Vandvik B* Relationship between measles virus-specific antibody activities and oligoclonal IgG in the central nervous system in patients with subacute sclerosing panencephalitis and multiple sclerosis. Med. Microbiol Immunol *1962* 63–72 1975
8. *Panelius M Salmi A A & Halonen P* Gel precipitation reactions between measles antigens and sera of patients with multiple sclerosis. Acta path. microbiol. scand. Sect. B *78* 588–592 1970
9. *Payne F E Baublis J V & Itabashi H H* Isolation of measles virus from cell cultures of brain from a patient with subacute sclerosing panencephalitis. New Engl. J Med. *281* 585–589 1969
10. *Salmi A Norrby E & Panelius M* Identification of different measles virus-specific antibodies in the serum and cerebrospinal fluid from patients with subacute sclerosing panencephalitis and multiple sclerosis. Infect. Immun. *6* 248–254 1972
11. *Schumacher G A Beebe G Kibler R F Kurland L T McDowell F, Nagler B, Sibley W A Tourtellotte W W & Willmon T L* Problems of experimental trials in multiple sclerosis. Report of a panel on "experimental trials of therapy in multiple sclerosis" N Y Acad. Sci *122* 552–568, 1965
12. *Strosberg, A D Karcher D & Lowenthal, A* Structural homogeneity of human subacute sclerosing panencephalitis antibodies. J Immunol. *115* 157–160 1975
13. *Vandvik B.* Immunopathological aspects in the pathogenesis of subacute sclerosing panencephalitis with special reference to the significence of the immune response in the central nervous system. Ann. clin. Res. *5* 308–315 1973
14. *Vandvik B* Oligoclonal IgG and free light chains in the cerebrospinal fluid of patients with multiple sclerosis and infectious diseases of the central nervous system. In press. Scand. J Immunol. 1977
15. *Vandvik B & Degré M.* Measles virus antibodies in serum and cerebrospinal fluid in patients with multiple sclerosis and other neurological disorders, with special reference to measles antibody synthesis within the central nervous system. J neurol. Sci. *24* 201–219, 1975
16. *Vandvik B Frøland S S., Stien R. & Degré M* Immunological features in a case of subacute sclerosing panencephalitis treated with transfer factor Scand J Immunol. *2* 367–374 1973
17. *Vandvik B & Norrby E* Oligoclonal IgG antibody response in the central nervous system to different measles virus antigens in subacute sclerosing panencephalitis. Proc. nat. Acad Sci (USA) *70* 1060–1063 1973
18. *Vandvik B Norrby E, Nordal H J & Degré M* Oligoclonal measles virus-specific IgG antibodies isolated from cerebrospinal fluids, brain extracts, and sera from patients with subacute sclerosing panencephalitis and multiple sclerosis. Scand. J Immunol. *5* 979–992 1976
19. *Zeman W & Kolar O* Reflections on the etiology and pathogenesis of subacute sclerosing panencephalitis. Neurology (Minneap.) *18* part 2, 1–7 1968.

Acta path. microbiol. scand. Sect. C, 85: 333–344, 1977

MIXED LYMPHOCYTE CULTURE REACTIONS AT DELIVERY AND IN THE PUERPERIUM EFFECTS OF PARITY, HLA ANTIGENS AND MATERNAL SERUM

Eija Herva and Anja Tiilikainen

Public Health Laboratory, Oulu, Finland and Finnish Red Cross Blood Transfusion Service, Helsinki, Finland

Herva, E. & Tiilikainen, A. Mixed lymphocyte culture reactions at delivery and in the puerperium. Effects of parity, HLA antigens and maternal serum. Acta path. microbiol. scand. Sect. C, 85: 333–344, 1977.

Mixed lymphocyte culture reactions between maternal and related neonatal cells at delivery and maternal and paternal cells about a week after delivery, and the effect of maternal serum and HLA antigens on these reactions were studied in 11 families with primiparous or secundiparous mothers and in 13 families with multiparous mothers (six or more pregnancies). Weak or absent MLC response of the mother to her infant was observed in one-third of primiparous and secundiparous mothers and in one-half of multiparous mothers. In some cases the non-reactivity could be due to genetic similarity, i.e. HLA or HLA-D identity between the mother and her infant. In other cases, this was obviously not a valid explanation and no apparent reason for the non-reactivity was found. The MLC suppressing effect of maternal serum on MLC reactions at delivery and about a week later was not correlated with the strength of maternal-neonatal MLC reaction. Four of the ten sera from multiparous mothers studied a week after delivery had an inhibitory effect of 50 per cent or more on MLC reactions involving stimulatory paternal cells. Responding paternal cells and other MLC combinations were also inhibited to varying degrees. None of the sera of primiparous and secundiparous mothers had an equally strong MLC inhibiting effect.

Key words: Mixed lymphocyte culture; delivery; puerperium.

E. Herva, Public Health Laboratory, Kajaanintie 46, 90100 Oulu 10, Finland

Received 12.ii.77. Accepted 12.ii.77.

Immunological hyporeactivity of the mother is one of the mechanisms proposed to explain the success of the foetus as an allograft (for discussion and references see *Beer & Billingham* 1971, *Billington* 1975, Lancet 1975).

In the mixed lymphocyte culture (MLC) reaction, which represents the sensitizing phase of allograft reaction *in vitro* (for review see *Häyry et al.* 1972, *Häyry* 1976), maternal cells respond to related neonatal cells (*Ceppellini et al.* 1971a), and the reaction produces cytotoxic lymphocytes (*Bon-*

be stated. The clarification of this question might shed light on virus or virus-related substances which appear to be immunogenic in the measles infection of the brain in patients with SSPE, but not in other conditions of measles immunization.

Grateful thanks are extended to Mrs. *Helle Dunker* and to Miss *Ruth Nilsen* for their excellent technical assistance. The support from the *Norwegian Research Council for Science and the Humanities* the *Oslo Multiple Sclerosis Society* and the *Norwegian Odd Fellow Order* is gratefully acknowledged

REFERENCES

1 *Connolly J H Allen I V Hurwitz L. J & Millar J H D* Measles virus antibody and antigen in subacute sclerosing panencephalitis. Lancet *I* 542–544 1967

2. *Dietzman D E Horta-Barbosa L., Krebs H., Maddew D L. Fucillo D A & Sever J L.* Diagnosis of subacute sclerosing panencephalitis by a simple spinal fluid gel precipitation test for measles. Pediatrics *49* 133–136 1972

3 *Horta Barbosa, L. Fucillo D A Sever J L. & Zeman W* Subacute sclerosing panencephalitis Isolation of measles virus from a brain biopsy Nature *221* 974 1969

4 *Karcher D van Sande M & Lowenthal A* Microelectrophoresis in agar gel of proteins of the cerebrospinal fluid and central nervous system. J Neurochem *4* 135–140 1959

5 *Laterre E. C* Les protéines du liquide céphalorachidien a l'état normal et pathologique Arscia, Bruxelles, 1965

6 *Norrby E & Hammarskjöld B* Structural components of measles virus Microbios *5* 17–29 1972

7 *Norrby E & Vandvik B* Relationship between measles virus-specific antibody activities and oligoclonal IgG in the central nervous system in patients with subacute sclerosing panencephalitis and multiple sclerosis. Med Microbiol. Immunol. *1962* 63–72 1975

8. *Panelius M Salmi A A & Halonen P* Gel precipitation reactions between measles antigens and sera of patients with multiple sclerosis. Acta path. microbiol. scand. Sect. B, *78* 588–592, 1970

9 *Payne F E Baublis J V & Itabashi H H* Isolation of measles virus from cell cultures of brain from a patient with subacute sclerosing panencephalitis. New Engl J Med *281* 585–589 1969

10 *Salmi A Norrby E. & Panelius, M* Identification of different measles virus-specific antibodies in the serum and cerebrospinal fluid from patients with subacute sclerosing panencephalitis and multiple sclerosis. Infect. Immun. *6* 248–254 1972.

11 *Schumacher G A Beebe G Kibler R. F Kurland L. T McDowell, F., Nagler B., Sibley W A Tourtellotte W W & Willmon T L.* Problems of experimental trials in multiple sclerosis. Report of a panel on experimental trials of therapy in multiple sclerosis" N Y Acad. Sci. *122* 552 568, 1965

12. *Strosberg A D., Karcher D & Lowenthal, A* Structural homogeneity of human subacute sclerosing panencephalitis antibodies J Immunol. *115* 157–160 1975.

13 *Vandvik B* Immunopathological aspects in the pathogenesis of subacute sclerosing panencephalitis with special reference to the significance of the immune response in the central nervous system. Ann. clin. Res. *5* 308–315, 1973

14 *Vandvik B.* Oligoclonal IgG and free light chains in the cerebrospinal fluid of patients with multiple sclerosis and infectious diseases of the central nervous system. In press. Scand. J Immunol 1977

15 *Vandvik B & Degré M* Measles virus antibodies in serum and cerebrospinal fluid in patients with multiple sclerosis and other neurological disorders, with special reference to measles antibody synthesis within the central nervous system. J neurol. Sci. *24* 201–219, 1975

16 *Vandvik B Frøland S S., Stien R. & Degré M* Immunological features in a case of subacute sclerosing panencephalitis treated with transfer factor Scand J Immunol. *2* 367 374 1973

17 *Vandvik B & Norrby E* Oligoclonal IgG antibody response in the central nervous system to different measles virus antigens in subacute sclerosing panencephalitis. Proc. nat. Acad Sci. (USA) *70* 1060–1063 1973

18. *Vandvik B Norrby E Nordal H J & Degré M* Oligoclonal measles virus-specific IgG antibodies isolated from cerebrospinal fluids, brain extracts, and sera from patients with subacute sclerosing panencephalitis and multiple sclerosis. Scand. J Immunol. *5* 979 992 1976.

19 *Zeman W & Kolar O* Reflections on the etiology and pathogenesis of subacute sclerosing panencephalitis. Neurology (Minneap.) *18* part 2, 1–7 1968.

HLA Dw2 homozygous cell that permitted typing for that determinant.

Cytotoxic Antibody Testing

Cytotoxic antibodies in maternal serum against paternal and neonatal cells were sought, using the microcytotoxicity method mentioned above.

RESULTS

MLC Reactions between Maternal and Neonatal Cells

Of the 11 *primiparous or secundiparous* mothers, seven responded significantly ($p < 0.01$) to the cells of their own infants (Table 3) Two of the four non-responding mothers did not stimulate the cells of their infants. Among primiparous mothers, non-responsiveness was less frequent (one of six) than among secundiparous mothers (three of five)

Six of the 12 multiparous mothers did not respond significantly to the cells of their infants in two of these mother-infant pairs non-responsiveness was reciprocal. Five of the seven mothers having been pregnant nine or more times were non-responsive to the cells of their infants (Table 4)

Neonatal cells responded to unrelated control cells two or even three times as strongly as maternal cells (Tables 3 and 4) Some of the infant cells stimulated weakly or not at all the cells of their multiparous mothers, and stimulated control cells also weakly even though responding strongly to them (Table 4) Time-course kinetics of maternal and neonatal MLC responses to each other did not differ from the kinetics of their responses to control cells in either group of families (not shown) Thus, no evidence of immune effector cell populations in the mothers was observed

Influence of HLA Antigens on MLC Reactions between Maternal and Neonatal Cells

In the group of *primiparous or secundiparous* mothers and their infants, the two reciprocally non-responsive mother infant pairs had identical HLA A and B antigens (Table 1 Families 1 and 11) In the other two pairs with maternal to neonatal non-responsiveness, the infant s cells responded to maternal cells. One of these infants (Table 1 Family 7) was homozygous for HLA Bw40. However the father's cells tested at the same time responded strongly to the cells of the infant, thus excluding HLA D homozygosity of the infant. In the two additional mother infant pairs with HLA B compatibility of the infant with the mother maternal and neonatal cells responded strongly to each other (Tables 1 and 3 Families 2 and 4)

None of the *multiparous mothers* (Table 2) was HLA identical with her infant. Two infants were HLA B compatible with their mothers (in Families 21 and 24 and perhaps also in Family 20) In family 21 both mother and infant were HLA Bw35 homozygous In these families, the mother did not respond to the cells of her infant. In family 24 the reaction was negative in the other direction also.

HLA Dw2 typing results did not explain weak or absent reactions between maternal and neonatal cells (Tables 2 and 4)

MLC Reactions between Parental Cells

Maternal cells responded to paternal cells in all six pairs in the first group and in all ten pairs in the second group. For the *primiparous and secundiparous mothers* the mean MF_x value (14479 ± 9756 CPM) did not differ significantly from the mean MC_x (12051 ± 4425 CPM) neither did the mean FM_x differ from the mean FC_x For the group of *multiparous mothers* the mean MF_x (3646 ± 1762 CPM) was significantly ($p < 0.01$) weaker than the mean MC_x (6740 ± 2663 CPM) the difference between the mean FM_x and the mean FC_x was almost significant ($p < 0.05$) However each experiment involving multiparous mothers included control cells from two persons, and the control eliciting the stronger response in each responding cell was selected as the reference cell for this particular combination, while results with only one control cell preparation

nard & Lemos 1972) However some mothers are clearly hyporesponsive or non responsive to the cells of their infants (*Ceppellini et al* 1971a *Herva* 1976)

Maternal serum in pregnancy and at term contains substances which inhibit MLC and other *in vitro* reactions of lymphocytes. These substances may be antibodies against paternal transplantation antigens (*Leventhal et al* 1970) or more generally non specific inhibitors which disappear soon after delivery (*Kasakura* 1972 *Leikin* 1972 *Herva & Jouppila* 1977)

In this study an explanation has been sought for the maternal hyporeactivity to related neonatal cells. Maternal and neonatal cells at delivery and maternal and paternal cells about a week after delivery were tested in one way MLC with and without maternal serum The responses were compared with the compatibility of their serologically defined HLA antigens and with the parity of the mother

MATERIALS AND METHODS

Eleven primiparous or secundiparous mothers (aged 20–37 mean 25 years) and 13 mothers with six or more pregnancies (27–45 mean 36 years) and their husbands and newborn infants were studied. Details of the parities and gravidities of the mothers are shown in the tables. One of the mothers (No 16) had severe pre-eclampsia and four (Nos. 14 18 20 and 21) mild pre-eclampsia associated in one case (No. 21) with essential hypertension. In addition one mother (No. 22) had essential hypertension All gave birth to full term infants, one of these being small for date (Family 16)

Samples of heparinized cord blood and first maternal blood were taken at delivery Paternal and second maternal blood samples were taken 4–12 days (mean 6.3 primiparous and secundiparous mothers) or 4–6 days (mean 5 1 multiparous mothers in one case in this group one day) after delivery Blood samples for control cells were from healthy unrelated donors, usually laboratory personnel.

Mononuclear cells were prepared (*Bøyum* 1968) as reported previously (*Herva* 1976 1977) Maternal serum for MLC inhibition and cytotoxicity testing was prepared simultaneously from clotted blood samples.

Mixed Lymphocyte Cultures

MLC experiments were performed using the microtitre plate method (*Hartzman et al* 1971) Technical details and observations on the culture conditions have been reported previously from our laboratory (*Herva* 1977) The first half of the experiments involving the primiparous and secundiparous mothers were performed on microtitre plates with flat-bottomed wells with 1.5×10^5 responding and irradiated (3000 rad) stimulating cells per well harvested on days four and six. The later half of the experiments (multiparous mothers) were performed using plates with round-bottomed wells and 0.5×10^5 of both component cells stimulating cells were irradiated with 6000 rad. These cultures were harvested on days three and five. Maternal serum was inactivated (56 C, 30 min) and diluted with the same amount of inactivated control serum (from non-transfused healthy male blood service volunteers) The final concentration of maternal serum in cultures was 10–12 per cent.

The MLC reaction mixtures will be referred to by abbreviations, where M = maternal cells, N = neonatal cells, F = paternal cells and C = control cells as responding population. When followed by x as subscript, they indicate irradiated stimulating cell populations. MLC reaction results are expressed as increments (or specific responses mean CPM of triplicate allogeneic cultures minus mean CPM of triplicate autologous cultures) and as relative responses (RR) calculated from the increment CPM values (for example $\frac{\text{CPM of } MN_x}{\text{CPM of } MC_x} \times 100$) When cells from two persons were used as controls, control cells inducing the stronger response served as reference cells in calculation of relative response Statistical significance of the weak MLC responses was evaluated using Student's t test and original CPM values without subtracting background

The effect of maternal serum on each MLC reaction was calculated using increment CPM values and the formula

$$\frac{\text{CPM in control serum minus CPM in maternal serum}}{\text{CPM in control serum}} \times 100$$

The direction of the effect is expressed by the signs—(= suppression) and + (= stimulation)

HLA Typing

The test used was a two-stage microcytotoxicity test (*Ames et al.* 1969) with 2–5 highly selected antisera for each antigen specificity Typing results of maternal neonatal and paternal cells permitted reliable genotyping in most families.

In the later part of this st dy nvolving multiparous mothers, most MLC experiments included a

Family No	Father HLA A; B C	Dw2 (RR)	Infant HLA A B C	Dw2 (RR)	Mother HLA A B; C	Dw2 (RR)
13	NT	NT	A9,Bw40 w6 / A2 Bw15 w6 Cw3	(100)	A3,B7 w6 / A2,Bw15 w6 Cw3	+ (16)
14	A1,B27 w4 Cw4 / A1 B8,w6	(60)	A1,B27 w4 Cw4? / A3,B7 w6	(38)	A3,Bw35 w6,Cw4 / A3,B7 w6	+ (10,3)
15	A2,Bw35,w6 / A10,Bw40 w6	(70)	A2,Bw35 w6 / A1,Bw37 w4 CT7	NT	A9,B12 / A1,Bw37 w4 CT7	+ (6)
16	Aw19,Bw40,w4 Cw3 / A3,Bw15,w6	(90)	Aw19 Bw40,w4 Cw3 / Aw25,B18,w6	(59)	Aw24,Bw16 w4 / Aw25,B18 w6	−(71;98)
17	NT	NT	A9 w25 B7 w16 w4 w6	NT	NT	NT
18	A3,B7 w6 / Aw19,Bw40 w6 Cw3	+ (1)	A3,B7 w6 / A3,Bw35,w4	+ (21)	A3,B27 w4 / A3,Bw35 w6	−(93;45)
19	A2,Bw15,Cw3 / A9 12	−(82)	A2,Bw15 Cw3 / A3,Bw35	−(100)	A1,B8 Cw4 / A3,Bw35	−(100 64)
20	NT	NT	A1,B8,w6	(65)	A3,B7 w6 / A1,B8,w6	−(52)
21	A3,9,B35 w6 CT7	−(54)	A3,9,Bw35 w6	NT	A9 11?,Bw35 w6	−(67)
22	A2,B12,w4 / Aw19;B? w6	−(50)	A2,B12,w4 / Aw19?,Bw35 w6 Cw4	NT	A3,B7 w6 / Aw19?;Bw35 w6 Cw4	+ (4)
23	A9 11,B7 w40 w6 Cw3	−(46)	NT	NT	A2,3,B5 7 w4 w6 Cw2,w1?	−(59)
24	A3,B7 w6 / A3,Bw16,w4	+ (8)	A3,B7 w6 / A9,Bw37 w4	(35)	Aw19,B7 6 / A9,Bw37 w4	−(59;89)
25	A1,B7 w6 / A3,B8,w6	+ (5)	A1 B7 w6 / A2,B5 w4	NT	A3;B7 w6 Cw3 / A2,B5 w4	+ (9,3)

a) RR = relative response to Dw2 homozygous typing cells; in six families maternal cells were typed for HLA Dw2 in two separate experiments.

TABLE 1 *HLA Antigens in the Families of Primiparous and Secundiparous Mothers*

Family No	Father HLA A,B	Infant HLA A,B	Mother HLA A,B
1	A2,B7/A3,B7	A2 B7/A9 B12	A2,B7/A9,B12
2	A2,B5/A3,Bw15	A2,B5/A28,B5	A9,B13/A28 B5
3	A11,B7/A11,B7	A11,B7/A3,Bw18	A9,Bw35/A3,Bw18
4	A2,B27/A3,Bw35	A2,B27/A28,B27	A3,Bw35/A28,B27
5	A3,Bw35/A9 Bw40	A3,Bw35/A3,B5	A11,B7/A3,B5
6	NT	NT	NT
7	A9,Bw40/A3,Bw15	A9,Bw40/Aw32,Bw40	A2,B5/Aw32,Bw40
8	A2,Bw15/Aw19,B12	A2,Bw15 A28,B5	A3,B7/A28,B5
9	NT	NT	NT
10	A2,9,Bw15,w40	NT	A2,28?,Bw15 w22
11	A2,3?,Bw22 w35	A2,3 Bw15 w35	A2,3,Bw15,35

NT = not tested.

were available for the experiments with primiparous and secundiparous mothers.

As shown in Tables 5 and 6 the mother's cells responded to the cells of the father even when they did not respond to the cells of the infant. In these cases the mean RR of MF_X was somewhat weaker than in the families with clearly positive MN_X reactions. Time-course kinetics of the responses of maternal and paternal cells to each other apparently did not differ from those of other reactions (not shown)

Effect of Maternal Serum on MLC Reactions

In maternal neonatal MLC experiments, 17 out of 18 maternal sera (the one exception being from a *multipara*) suppressed to varying degrees all the MLC combinations in the experiment. The mean suppressive effect was 54 per cent in the group of six primiparous/secundiparous mothers and 34 per cent in the 11 multiparous mothers. The effect could of course, not be evaluated in MLC negative or very weakly positive combinations, i. e. in a large proportion of maternal neonatal MLCs Because of this fact and the apparent nonspecificity of the effect no attempt was made to evaluate its specificity in maternal neonatal MLC experiments.

In addition maternal sera taken about a week after delivery usually had a suppressive effect on most MLC combinations in both groups of mothers (Tables 5 and 6) However in multiparous mothers, many sera enhanced the reactions and in four cases the mean effect was stimulating rather than suppressing (Table 6)

Suppression of the increment CPM by 50 per cent or more was considered arbitrarily to indicate "clear inhibition In the first group, no serum had such an effect in the second group four of the ten sera studied caused clear inhibitions. The strongest effect of the serum was usually seen in combinations involving stimulating paternal cells (F_X) (Table 6) This was seen most clearly in family 21 where the mean inhibition in the F_X combinations was 92 per cent, in other combinations only 12 per cent (the reactions with Dw2 homozygous typing cells, not shown in the table were included in these calculations) In families 16 19 and 25 also any F but notably FC_X combinations were markedly inhibited. In family 19 maternal serum was cytotoxic against paternal cells but not against control or Dw2 homozygous typing cells that were included in the MLC experiment in this case. It inhibited all these cells as stimulating cells (in most cases perhaps also as responding cells reactions of Dw2 homozygous cells not shown in the tables)

TABLE 2 HLA Antigens the Families of 34 Uniparous Mothers

Family No	Father HLA A; B; C	Dw2(RR)	Infant HLA A; B; C	Dw2(RR)	Mother HLA A; B; C	Dw2(RR)
13	NT	NT	A9;Bw40 w6	(100)	A3,B7 w6	+ (16)
			A2 Bw15 w6 Cw3		A2,Bw15 w6 Cw3	
14	A1 B27 w4 Cw4	(60)	A1,B27 w4 Cw4	?(38)	A3,Bw35 w6 Cw4	+ (10;3)
	A1 B8,w6		A3,B7 w6		A3 B7 w6	
15	A2,Bw35,w6	(70)	A2,Bw35 w6	NT	A9,B12	+ (6)
	A10,Bw40 w6		A1,Bw37 w4 CT7		A1,Bw37 w4 CT7	
16	Aw19,Bw40 w4 Cw3	(90)	Aw19,Bw40 w4 Cw3	(59)	Aw24,Bw16,w4	−(71;98)
	A3,Bw15 w6		Aw25,B18,w6		Aw25,B18 w6	
17	NT	NT	A9 w25 B7 w16 w4 w6	NT	NT	NT
18	A3,B7 w6	+ (1)	A3,B7 w6	+ (21)	A3,B27 w4	(93 45)
	Aw19,Bw40,w6;Cw3		A3 Bw35,w6		A3,Bw35 w6	
19	A2,Bw15,Cw3	−(82)	A2,Bw15 Cw3	−(100)	A1,B8 Cw4	−(100 64)
	A9;12		A3,Bw35		A3,Bw35	
20	NT	NT	?	−(85)	A3,B7 w6	−(32)
			A1,B8,w6		A1,B8 w6	
21	A3,9,B35,w6 CT7	(54)	A3,9,Bw35 w6	NT	A9 11?,Bw35 w6	−(67)
22	A2,B12,w4	−(50)	A2,B12 w4	NT	A3,B7 w6	+ (4)
	Aw19,B w6		Aw19?,Bw35 w6 Cw4		Aw19?,Bw35 w6 Cw4	
23	A9 11,B7 w40 w6 Cw3	−(46)	NT	NT	A2,3;B5 7 w4 w6 Cw2,w1?	−(59)
24	A3,B7 w6	+ (8)	A3,B7 w6	?(55)	Aw19,B7 w6	−(59 89)
	A3,Bw16 w4		A9;Bw37 w4		A9;Bw37 w4	
25	A1,B7 w6	+ (5)	A1 B7 w6	NT	A3,B7 w6 Cw3	+ (9,3)
	A3,B8,w6		A2 B5 w4		A2 B5 w4	

) RR = relative response to Dw2 homozygous typing cells; i six families maternal cells were typed for HLA Dw2 in two separate experiments.

TABLE 1 *HLA Antigens in the Families of Primiparous and Secundiparous Mothers*

Family No.	Father HLA A,B	Infant HLA A B	Mother HLA A,B
1	A2 B7/A3,B7	A2,B7/A9,B12	A2,B7/A9,B12
2	A2 B5/A3,Bw15	A2,B5/A28 B5	A9,B13/A28,B5
3	A11,B7/A11,B7	A11 B7/A3 Bw18	A9,Bw35/A3,Bw18
4	A2,B27/A3 Bw35	A2,B27/A28,B27	A3,Bw35/A28,B27
5	A3,Bw35/A9,Bw40	A3,Bw35/A3,B5	A11,B7/A3,B5
6	NT	NT	NT
7	A9,Bw40/A3,Bw15	A9,Bw40/Aw32,Bw40	A2,B5/Aw32,Bw40
8	A2,Bw15/Aw19,B12	A2,Bw15/A28,B5	A3,B7/A28,B5
9	NT	NT	NT
10	A2,9,Bw15 w40	NT	A2,28?,Bw15 w22
11	A2,3?,Bw22 w35	A2,3,Bw15 w35	A2,3 Bw15,35

NT = not tested.

were available for the experiments with primiparous and secundiparous mothers.

As shown in Tables 5 and 6 the mother's cells responded to the cells of the father even when they did not respond to the cells of the infant. In these cases the mean RR of MF_x was somewhat weaker than in the families with clearly positive MN_x reactions. Time-course kinetics of the responses of maternal and paternal cells to each other apparently did not differ from those of other reactions (not shown)

Effect of Maternal Serum on MLC Reactions

In maternal neonatal MLC experiments, 17 out of 18 maternal sera (the one exception being from a multipara) suppressed to varying degrees all the MLC combinations in the experiment. The mean suppressive effect was 54 per cent in the group of six primiparous/secundiparous mothers and 34 per cent in the 11 multiparous mothers. The effect could of course not be evaluated in MLC negative or very weakly positive combinations, i. e in a large proportion of maternal neonatal MLCs Because of this fact and the apparent nonspecificity of the effect, no attempt was made to evaluate its specificity in maternal neonatal MLC experiments.

In addition, maternal sera taken about a week after delivery usually had a suppressive effect on most MLC combinations in both groups of mothers (Tables 5 and 6) However in multiparous mothers, many sera enhanced the reactions, and in four cases the mean effect was stimulating rather than suppressing (Table 6)

Suppression of the increment CPM by 50 per cent or more was considered arbitrarily to indicate "clear inhibition" In the first group no serum had such an effect in the second group four of the ten sera studied caused clear inhibitions The strongest effect of the serum was usually seen in combinations involving stimulating paternal cells (F_x) (Table 6) This was seen most clearly in family 21 where the mean inhibition in the F_x combinations was 92 per cent, in other combinations only 12 per cent (the reactions with Dw2 homozygous typing cells, not shown in the table, were included in these calculations) In families 16 19 and 25 also any F but notably FC_x combinations were markedly inhibited. In family 19 maternal serum was cytoxic against paternal cells but not against control or Dw2 homozygous typing cells that were included in the MLC experiment in this case. It inhibited all these cells as stimulating cells (in most cases perhaps also as responding cells reactions of Dw2 homozygous cells not shown in the tables)

TABLE 5 *MLC Response of Maternal [illegible] Neonatal and Paternal Cells and the Effect [illegible] Maternal S[illegible]rum on MLC Reactions between Maternal, Paternal and Control C[illegible]lls in Prim[illegible]par[illegible]us and S[illegible]c[illegible]d[illegible]parous Mother*

Family No.	Par Grav	MN RR	MF RR	MF_x	MC_x	FM_x	FC_x	CM_x	CF_x	mean	Cytotox. antibod.
				Effect of maternal serum on MLC reactions[b]							
1	I/I	(−0)	49	—15	+10	NT	—25	—14	—19	—13	—
11	II/III	(0)	23	—8	—15	—48	—14	—8	—33	—21	—
7	I/II	(4)	97	+8	5	—33	—6	—34	—9	—13	—
3	I/I	62	188	—10	+8	+22	—5	—21	—11	—3	—
10	II/II	86	+ +	—23	?[d]	—34	—36	—34	—28	—31	(weak)
5	I/I	+ +	64	+10	—21	—29	—18	—27	—36	—20	—

a) MLC responses of maternal [illegible] neonatal (MN_x) and paternal (MF_x) cells tested [illegible] separate experiments, MN_x [illegible]t delivery MF_x about a week later and results expressed as relat[illegible] response (RR)

b) Effect of maternal serum tested about a week after d[illegible]li[illegible]ery [illegible]n an experiment including cells from mother father and one or two controls; for further explanations see Table 3 and text.

c) + + = strong reaction, adequate control for calculation of relative response lacking.

d) ? = typing response effect of maternal serum not evaluated.

e) () = statist[illegible]cally not significant reaction ($p > 0.01$)

TABLE 3 *MLC Reactions between Maternal Neonatal and Control Cells in Primiparous and Secundiparous Mothers*

Family No.	Par Grav	MN_x CPM[b]	RR	MC_x CPM	NM_x CPM	RR	NC_x CPM	CN_x CPM	CM_x CPM
1	I/I	(−9)[d]	(−0)	5781	(5351)	(16)	34296	9960	8515
2	I/I	5842	36	16086	12140	20	60593	26205	10671
3	I/I	2478	62	4007	9680	24	40687	16631	2936
4	I/I	9682	NT	NT	7287	NT	NT	NT	NT
5	I/I	5406	NT	NT	9639	NT	NT	NT	NT
6	I/I	809	13	6109	7969	82	9734	9186	6450
7	I/II	(458)	(4)	10758	8396	21	40881	14662	5320
8	II/II	718	12	6130	10890	26	41155	13240	5131
9	II/II	(710)	(24)	2953	9709	40	24062	24671	8398
10	II/II	5792	86	6748	NT	NT	19997	10695	9929
11	II/III	(53)	(0)	14632	(945)	(6)	16334	8033	11872

a) M = maternal, N = neonatal, C = control cells responding, M_x N_x, C_x = stimulating cells.
b) the mean CPM (counts per minute) of triplicate cultures as increment.
c) RR = relative response.
d) () = statistically not significant reaction ($p > 0.01$)
e) NT = not tested.

TABLE 4 *MLC Reactions between Maternal Neonatal and Control Cells in Multiparous Mothers*

Family No	Par Grav	MN_x CPM[b]	RR	MC_x CPM	NM_x CPM	RR	NC_x CPM	CN_x CPM	RR	CM_x CPM	RR
13	VI/VI	(375)[d]	(9)	4084	5249	56	9338	4234	45	4830	68
14	VI/VI	668	17	4029	2141	39	5443	(868)	(23)	(1196)	(31)
15	VIII/X	1356	27	4959	3130	40	7848	3114	NT	2868	NT
16	VI/VII	3151	45	6986	5217	27	20518	7515	136	8745	159
17	VIII/VIII	3724	31	11862	12133	52	23261	3736	NT	6108	NT
18	VIII/VIII	2059	111	1849	7211	88	8221	8840	154	5012	87
19	VIII/IX	(509)	(14)	3537	2834	37	7760	2832	51	4562	81
20	IX/X	(88)	(2)	3779	(1426)	(20)	7118	(1084)	(23)	(829)	(17)
21	X/XIV	(59)	(4)	1386	1609	35	4629	(215)	NT	(888)	NT
22	XI/XI	1785	30	5989	7690	36	21390	4185	NT	1674	NT
23	XI/XII	(293)	(5)	5981	6998	42	16481	(289)	NT	2077	NT
24	XIII/XIV	(14)	(1)	2523	(−462)	(−4)	11550	1879	61	2491	82

For expl nations see T ble 3

In two families (14 and 18) reactions other than those with F_x were suppressed more strongly but the suppression was usually not over 50 per cent. In family 18, maternal serum suppressed the responses of maternal and control cells to Dw2 homozygous cells by 44 and 59 per cent, respectively (not shown) In the three combinations with F_x the mean suppression was only 23 per cent. In this family cells of the father and of the infant were positive (heterozygous) for Dw2, and the mother was negative.

The suppressive effect of maternal serum did not depend on the strength of maternal MLC response to neonatal cells, whether the suppression was studied in experiments between maternal and neonatal or between maternal and paternal cells. This is shown in Table 5 and 6, where the results are arranged according to the strength of maternal neonatal MLC response.

DISCUSSION

We tried to find explanations for the very weak or absent MLC responses of the mother's cells to the cells of her newborn infant, a situation discovered in a considerable proportion of cases (*Ceppellini et al* 1971a, *Jenkins & Curzen* 1974 *Lawler et al* 1975 *Herva* 1976) In the present study the response was defined as negative when neonatal cells did not induce a significantly ($P < 0.01$) increased DNA synthesis compared to the autologous control values. When evaluated in this way 36 per cent of the primiparous or secundiparous mothers and 50 per cent of the multiparous mothers were non-responsive to the infant's cells. Maternal responses to neonatal cells were weak (relative response under 30) in two additional families in both groups

According to the results presented in this paper and in other studies (*Ceppellini et al.* 1971a, *Leikin* 1972, *Kasakura* 1972, *Carr et al* 1974 *Herva & Jouppila* 1977) the cells of pregnant and postpartum women react with allogeneic cells in MLC, though less vigorously than cells from non-pregnant subjects (*Ceppellini et al.* 1971a, *Jones & Curzen* 1973) Cells from newborn infants responded in MLC more vigorously than cells from adult subjects, as also documented earlier (*Ceppellini et al.* 1971a, *Herva* 1976) and their stimulating capacity was generally comparable to that of adult cells. In the group of multiparous mothers, neonatal cells had a weakly stimulating effect on both maternal and control cells in some cases. This could be due to occasional genetic similarity between the cells, as only one or two control cell populations were used in each experiment (c. f Families 20 and 21 in Table 4)

In most cases, however neonatal cells stimulated control cells even when not stimulating maternal cells. Thus, the weak or absent responses of maternal cells to neonatal cells and sometimes also neonatal to maternal cells cannot be explained by non-specifically low MLC stimulatory capacity of these cells.

In some cases, maternal-neonatal MLC non reactivity is readily even though indirectly explained according to the present knowledge of the human major histocompatibility (HLA) system (for review see *Bach & van Rood* 1976) When a parent and a child are identical for serologically defined HLA antigens, about a half of the pairs are also reciprocally MLC negative (*Dausset et al* 1973) This situation was exemplified in the present study by families 1 and 11 The HLA B antigens in these families, B7 (inherited from the father in family 1) Bw15 and Bw35 are positively associated with HLA D determinants Dw2, Dw4 and Dw1 respectively (*Jersild et al* 1973 *Thomsen et al.* 1975 *Thorsby et al.* 1975) Thus in these families, the mother and her infant possibly also had identical HLA D antigens. Moreover in family 21 the mother and the infant, homozygous of HLA Bw35 could also be homozygous for Dw1 The mother did not respond to the infant's cells, and the reaction was weak in the other direction also The RR of 35 possibly resulted from the fact that the control cells also had Bw35 and did not respond significantly to the cells from

TABLE 6 *MLC Responses of Maternal to Neonatal and Paternal Cells and the Effect of Maternal Serum on MLC Reactions between Maternal Paternal and Control Cells in Multiparous Mothers*

Family No.	Par Grav	MN_x[a] RR	MF_x RR	Effect of maternal serum on MLC reactions[b]							Cytotox. antibod.
				MF_x	MC_x	FM_x	FC_x	CM_x	CF_x	mean	
24	VIII/XIV	(1)	57	—5	—1	+18	+9	+28	—1	+8	—
21	X/XIV	(4)	46	—91	—28	?d	—23	—23	—90c	—51	—
23	XI/XII	(5)	20	+18	—11	+35	—10	+10	—10	+5	—
19	VIII/IX	(14)	46	—93c	—76c	?d	—71c	+77	—94c	—51	+
14	VI/VI	17	96	6	—47	—5	—47	—33	—22	—25	—
15	VIII/X	27	30	+55	+31	—9	+19	+13	—8	+17	—
22	XI/XI	30	93	+24	+4	+6	+14	—11	—2	+6	—
16	VI/VII	45	124	—66c	—56	—27	—36	—22	—58c	—44	—
18	VIII/VIII	111	46	—10	—10	—46	—39	+35	—40	—18	—
25	VI/IX	NT	54	—57	—37	—64c	—74c	—?	—66c	—53	—

a) and b) for explanations see Table 5
c) clear inhibition Inhibitory effect of maternal serum over 50 per cent.
d) ? = typing response effect of maternal serum not evaluated.
e) () = statistically not significant reaction.

cytotoxicity of the serum. In addition, *Robert et al.* demonstrated that the inhibitory activity behaved as IgG in chromatography and in microfiltration or high-speed centrifugation the sera showed non-particulate nature

Human pregnancy seems to be a suitable stimulus for the formation of MLC inhibiting B-lymphocyte antibodies. One possible explanation for this may be that B-lymphocytes more often than T lymphocytes cross the placenta from the foetus to the mother (*Schröder* 1975) especially as placental cells are devoid of HLA D determinants (*Goodfellow et al.* 1976) However these antibodies are not formed regularly Strong MLC reaction blocking activity is more an exception than a rule in primiparous or secundiparous mothers (*Herva & Jouppila* 1977)

This study was primarily an attempt to find possible explanations for the MLC non reactivity sometimes observed between maternal and neonatal cells However we succeeded best in demonstrating the difficulties encountered when outbred individuals such as human beings are studied by a biologically variable and technically poorly standardizable method such as MLC reaction in the irrecoverable situation of birth. Transplantation immunology of human pregnancy should be studied further using MLC with multiple control cells of each kind (maternal, unrelated male, neonatal) Furthermore, carefully planned experiments are necessary in conjunction with other tests such as cell-mediated cytotoxicity both direct and antibody dependent, B-cell antibody determination, and some method for HLA D typing of all the cells studied, if neonatal cells are ever available in sufficient quantities.

We thank Docent *Pentti J ppila*, M.D Department of Obstetrics and Gynaecology University of Oulu, for his help in collecting the blood samples and the obstetrical and family data of the subjects. Our sincere thanks are due to Mrs *Marja Salonen* and *Kaisa Järvenpää* for their excellent technical assistance, and to Miss *Ruth Leukko* for her help in typing the manuscript

REFERENCES

Albrechtsen D Solheim B. G & Thorsby E. Antiserum inhibition of the mixed lymphocyte culture (MLC) interaction. Inhibitory effect of antibodies with HLA-D associated determinants. Cell. Immunol. *28* 258–273 1977

Amos D B., Bashir H., Boyle W., MacQueen, M & Tiilikainen, A. A simple microcytotoxicity test. Transplantation *7* 220–222 1969

Bach F H & van Rood J J. The major histocompatibility complex (Second of three parts) New Engl J Med. *295* 872–878, 1976.

Beer A E & Billingham R. E. Immunobiology of mammalian reproduction. Adv Immunol *14* 1–84 1971

Billington W D. Immunological aspects of normal and abnormal pregnancy Eur Obstet. Gynec. Reprod Biol. *5* 147–154 1975.

Bondevik H & Thorsby E. The human mixed lymphocyte culture (MLC) interaction after in vivo alloimmunization. I Kinetics. Cell. Immunol. *11* 409–411 1974

Bonnard G D & Lemos L. The cellular immunity of mother versus child at delivery Sensitization in unidirectional mixed lymphocyte culture and subsequent ⁵¹Cr-release cytotoxicity test. Transplant. Proc. *4* 177–180 1972

Böyum A Isolation of mononuclear cells and granulocytes from human blood. Scand. J clin. Lab. Invest. *21* suppl. 97 77–89 1968.

Carr M C., Stites D P & Fudenberg, H H Cellular immune aspects of the human fetal-maternal relationship. III Mixed lymphocyte reactivity between related maternal and cord blood lymphocytes. Cell. Immunol. *11* 332–341 1974

Ceppellini, R., Bonnard G D Coppo F., Magliano V C & Pospisil M. Mixed leukocyte culture and HL-A antigens. I Reactivity of young fetuses, newborns and mothers at delivery Transplant. Proc. *3* 58–63 1971a.

Ceppellini R., Bonnard C D., Coppo F Magliano V C Pospisil, M., Curtoni, E. S & Pellegrino M Mixed leukocyte cultures and HL A antigens. II. Inhibition by anti-HL-A sera. Transplant. Proc. *3* 63–70 1971b.

Charmot D Mawas C. E., Legrand L. & Sasportes M Secondary response of in vitro primed human lymphocytes to allogeneic cells V The secondary proliferation and CML response of in vitro primed lymphocytes have the same immunogenetic requirements as in vitro primed cells. Immunogenetics *3* 157–165 1976.

Dausset J Sasportes M & Lebrun A. Mixed lymphocyte cultures between serologically HL-A

the mother or the infant. In this family paternal cells responded weakly (typing response *Jorgensen et al* 1973) to maternal cells, which supports the HLA D homozygosity suggested above.

Possibilities for typing more HLA D determinants are clearly needed in order to elucidate further this aspect of the problem. HLA D typing (with homozygous typing cells) of the cells from newborn infants perhaps requires the double standardization procedure (*Ryder et al* 1975) more than healthy adult cells. Our results in families 14 and 24 might indicate that neonatal cells respond to homozygous typing cells relatively more vigorously than adult cells, just as they generally respond more vigorously in MLC than adult cells

Our experimental plan included time-course kinetics of MLC reactions, and there was no evidence of early peak response of *in vivo* sensitized lymphocytes (*Bondevik & Thorsby* 1974 *Charmot et al* 1976) which would have explained the low responses at later harvest time

Evidently MLC experiments performed during the first postpartum week between the paternal cells do not help in evaluating the possible causes of maternal neonatal non reactivity In both groups all the mother's cells responded to the cells of her husband and in those families where MN_x was absent, MF_x mean values were lower than in the families with normal MN_x responses This may be due to similarities of the HLA system between mother and father and/or to some pregnancy-associated modification in maternal reactivity In the group of multiparous mothers, parental cells responded less vigorously to each other than to unrelated cells. This may again be due to genetic similarity between the parents in this (whole) group in addition to possible pregnancy induced effects. These large families were mostly from rural communities in Northern Finland There is a degree of genetic isolation in these rural areas (*Nevanlinna* 1972) probably leading to an abnormally small degree of HLA polymorphism in the large families studied.

There was no apparent correlation between the strength of MLC responses of maternal cells to related neonatal cells at delivery and the MLC reaction inhibiting effect of maternal serum whether this effect was evaluated at term or four to six days later This is contrary to the findings of *Jones & Carter* (1974) who suggested this kind of straight correlation on the basis of a smaller number of experiments.

The mean suppressive effect of maternal serum at delivery was even stronger in primiparous or secundiparous mothers than in multiparous mothers. Because of the non-specific suppression (*Kasakura* 1972 *Leikin* 1972 *Herva & Jouppila* 1977) maternal sera at delivery may be unsuitable when MLC inhibiting antisera are sought.

Cytotoxic antisera with serologically defined HLA antigen specificities inhibit MLC reactions when either responder or stimulator cells have the relevant specificities (*Ceppellini et al.* 1971b *Thorsby & Solheim* 1973) Antibodies reacting with B lymphocytes specifically inhibit their stimulating function in MLC (*van Leeuwen et al* 1973 *Winchester et al* 1975 *Wernet* 1976) Our findings are consistent with these observations, even though the effect of sera with no HLA A B, C antibodies on responding cells was not excluded in some experiments. The finding that as stimulating cells, homozygous typing cells were inhibited more strongly than paternal cells heterozygous for the same specificity agrees with the observations of *Albrechtsen et al* (1977) This would merit confirmation using a larger number of sera from multiparous women and a representative panel of typing cells.

Our results are similar to those reported by *Robert et al* (1973) in a more extensive and thorough study These similarities are (1) high frequency of MLC inhibiting antibodies in the sera of multiparous mothers (2) inhibition of paternal cells as both responder and stimulator cells though more often and more strongly as stimulator cells, and (3) association of inhibiting activity sometimes, but not usually with complement dependent

Acta path. microbiol. scand. Sect. C, 85 345–349 1977

LIMITING FACTORS IN BACTERIAL PHAGOCYTOSIS BY HUMAN POLYMORPHO NUCLEAR LEUKOCYTES

B. Björkstén, P. K. Peterson, J. Verhoef and P. G. Quie

Department of Paediatrics, University of Umeå, 901 85 Umeå, Sweden, and University of Minnesota, Minneapolis MN 55455 U.S.A.

Björkstén, B., Peterson, P. K., Verhoef, J. & Quie, P. G. Limiting factors in bacterial phagocytosis by human polymorphonuclear leukocytes. Acta path. microbiol. scand. Sect. C, 85 345–349 1977

Limiting factors in neutrophil phagocytosis were studied using a sensitive assay by which attachment, ingestion and intracellular killing of bacteria could be separated. Phagocytosis was found to be limited by the attachment capacity of neutrophils. Ingestion and intracellular killing proceeded at constant rate proportional to the number of bacteria attached to the neutrophils.

Key words: Bacterial phagocytosis; limiting factors; human polymorphonuclear leukocytes.

B. Björkstén, Department of Paediatrics, University of Umeå, 901 85 Umeå, Sweden.

Received 22.iv.77 Accepted 22.iv.77

An intact phagocytic system is critical for adequate host defense against bacterial infection. Studies in recent years have revealed defective phagocyte function in several groups of patients with an increased susceptibility to infections (1). Evaluation of phagocyte function is an important part of an investigation of patients with recurrent infections. However the phagocytic process is complex and influenced by numerous extra- and intracellular factors (1). Laboratory methods used in the study of phagocyte function are poorly standardized, making comparison of results obtained in different laboratories difficult. By most methods the total phagocytic event, i.e. ingestion and the process of intracellular killing of bacteria are judged on the basis of the loss of viable microbes (4–6). Refinements of technique have been aimed at defining separately the ingestion and killing phases of phagocytosis, e.g. by adding phenylbutazone to the phagocytic mixtures (7). Methods based on viable counts, however are not sensitive enough to demonstrate subtle variations in the phagocytic process.

By combining a sensitive assay which measures ingestion of *radio-labelled bacteria* with a test method based on the loss of viable microbes, we have studied limiting factors in the phagocytic process separating attachment, ingestion and intracellular killing. In this study we found that phagocytosis is limited by the number of bacteria that can be attached to the cells and that killing is proportional to the number of bacteria phagocytized.

MATERIAL AND METHODS

Bacterial strains. Serum resistant strains of *Escherichia coli* 022 H16, beta haemolytic streptococcus group B type 1C, *S. aureus*, Cowan 1 strain and a clinical isolate of *S. epidermidis* were used. The

identical parent and child and between HL A homozygous and heterozygous individuals. Transplant. Proc. *5* 1511–1515 1973

Goodfellow P N Barnstable C J Bodmer W F Snary D & Crumpton M J Expression of HLA system antigens on placenta Transplantation *22* 595–603 1976

Hartzman R. J Segall M., Bach M L. & Bach F H Histocompatibility matching VI Miniaturization of the leukocyte culture test A preliminary report. Transplantation *11* 268–273 1971

Herva E. Mixed lymphocyte culture reactivity of neonatal lymphocytes. Lancet *I* 919–920 1976

Herva E. Comparison of microtitre plates with flat-bottomed and round-bottomed wells for mixed lymphocyte culture (MLC) Acta path. microbiol. scand Sect. C, *85* 90–98 1977

Herva E & Jouppila P Mixed lymphocyte culture reactions between parental cells in pregnancy and puerperium. Acta path. microbiol. scand. Sect. C *85* 99–106 1977

Häyry P *Anamnestic responses in mixed lymphocyte* culture induced cytolysis (MLC-CML) reaction. Immunogenetics *3* 417–453 1976

Häyry P Andersson L. C Nordling S & Virolainen M Allograft response in vitro. Transplant Rev *12* 91–140 1972.

Jenkins D M & Good S Mixed lymphocyte reaction and placentation Nature New Biol. *240* 211–212 1972.

Jersild C Hansen G S Svejgaard A Fog T Thomsen M & Dupont B Histocompatibility determinants in multiple sclerosis, with special reference to clinical course. Lancet *II* 1221–1225 1973

Jones E & Curzen P The immunological reactivity of maternal lymphocytes in pregnancy J Obstet. Gynec. Br Commonw *80* 608–610 1973

Jones E & Curzen P Blocking one-way maternal-foetal MLR. Nature *250* 791 1974

Jorgensen F Lamm L. U & Kissmeyer Nielsen F Mixed lymphocyte cultures with inbred individuals An approach to MLC typing Tissue Antigens *3* 323–339 1973

Kasakura S The inhibitory effect of pregnant plasmas on the reactivity of mixed leucocyte cultures. *In Schwarz M R* (Ed.) Proc Sixth Leucocyte Culture Conference. Academic Press, New York and London 1972 p 711–723

Lancet The fetus as a homograft. Lancet *II* 535–536 1975

Lawler S D Ukaejiofo E. O & Reeves B R Interaction of maternal and neonatal cells in mixed lymphocyte cultures. Lancet *II* 1185–1187 1975

van Leeuwen A Schuit H R E. & van Rood, J J Typing for MLC (LD) II The selection of nonstimulator cells by MLC inhibition tests using SD-identical stimulator cells (MISIS) and fluorescence antibody studies. Transplant. Proc. *5* 1539–1542, 1973

Leikin S The immunosuppressive effect of maternal plasma. In *Schwarz M R.* (Ed.) Proc. Sixth Leucocyte Culture Conference Academic Press New York and London 1972 p. 725–742

Leventhal B G., Buell D Yankee R., Rogentine G N Jr & Terasaki P., The mixed leucocyte response effect of maternal plasma. In *Harris, J E* (Ed.) Proc. Fifth Leucocyte Culture Conference Academic Press, New York, 1970, p 473–486

Nevanlinna H R The Finnish population structure A genetic and genealogical study Hereditas *71* 195–236 1972.

Robert M Betuel H & Revillard J P., Inhibition of the mixed lymphocyte reaction by sera from multipara. Tissue Antigens *3* 39–56, 1973

Ryder L. P Thomsen M Platz P & Svejgaard A Data reduction in LD-typing. In *Kissmeyer Nielsen F* (Ed.) Histocompatibility Testing 1975 Munksgaard Copenhagen 1975, p 557–562

Schröder J Are fetal cells in maternal blood mainly B lymphocytes? Scand. J Immunol. *4* 279–285 1975

Thomsen M Platz P., Ortved Andersen O Christy M Lyngsøe J Nerup J Rasmussen K Ryder L. P Staub Nielsen L. & Svejgaard A MLC typing in juvenile diabetes mellitus and idiopathic Addison's disease. Transplant. Rev *22* 125–147 1975

Thorsby E Helgesen A Rankin B., Möller E. & Kaakinen A Identification of five lymphocyte activating determinants in man. Tissue Antigens *6* 147–160 1975

Thorsby E. & Solheim B. G Specificity of antibodies that inhibit the MLC response. Transplant Proc. *5* 1859–1862 1973

Wernet P Human Ia-type alloantigens method of detection, aspects of chemistry and biology markers for disease states. Transplant. Rev *30* 271–298 1976

Winchester R J., Fu S M Wernet P., Kunkel H G Dupont B & Jersild C Recognition by pregnancy serums of non HL-A alloantigens selectively expressed on B lymphocytes. *J Exp* Med. *141* 924 929 1975

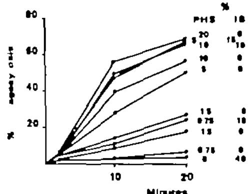


Fig 2 Phagocytosis of *E. coli* in mixtures of varying concentrations of PHS and heat-inactivated serum (IS) after 2, 10 and 20 min tes incubation. The bacteria to PMN ratio was 10 1

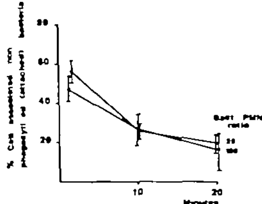


Fig 3 Proportion of cell-associated, non-ingested bacteria at two different bacteri to PMN ratios. The figures show the percentage of cell-associated, non-ingested staphylococci after 2, 10 and 20 minutes incubation. Mean and range of four experiments.

Using lysostaphin, the attachment and ingestion phases of the phagocytic process could be evaluated separately for phagocytosis of *S aureus* In Fig 3 the number of extracellular cell-associated bacteria is expressed as a percentage of the total number of cell associated bacteria As shown in this figure the proportion of cell-associated non-ingested bacteria was similar for two different bacteria to PMN ratios (20 1 and 100 1) thus indicating that once bacteria were attached to the cells they were ingested at a constant rate

The killing rate of ingested bacteria was not influenced by variations in bacteria to PMN ratio from 2 1 to 100 1 i.e. varying absolute numbers of ingested bacteria did not affect intracellular killing (Fig 4) After 2 minutes incubation, 6–9 per cent of the cell-associated bacteria were alive, after 10 minutes 1 4–4 per cent and after 20 minutes 1.2–2 5 per cent were viable, independent of the bacteria to PMN ratio. The results were similar for all four bacterial species tested.

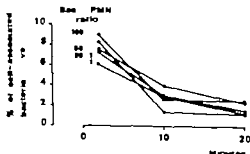


Fig 4 Effect of varying bacteria to PMN ratios on the number of alive cell-associated *E. coli* bacteria after 2, 10 and 20 minutes incubation.

DISCUSSION

We have studied PMN phagocytosis of four different bacterial species and found that although the total number of phagocytized bacteria increased with an increase in the bacteria to PMN ratio, the proportion of ingested microorganisms at 10 and 20 minutes remained constant for each species for bacteria to PMN ratios between 1 1 and 20 1 (Fig. 1) At higher ratios, the proportion of phagocytized bacteria decreased for all the four species, although the total number of ingested bacteria continued to increase. These results indicate that within a rather wide range, the bacteria to PMN ratio is not a critical factor for phagocytosis.

Dilution of serum opsonins was found to be critical for phagocytosis The concentration of heat-stable opsonins was lower than that of heat labile opsonins, since opsonization of *E. coli* or streptococci in diluted serum could be improved by the addition of heat inacti-

E. coli and *S. epidermidis* strains were known to activate complement via the alternative pathway while the streptococcal and *S. aureus* strains lacked this capacity. The bacteria were cultured in Mueller Hinton broth containing 2 μCi of ^{3}H thymidine per ml for 12 to 16 hours and then washed in phosphate-buffered saline (PBS) pH 7.4 and diluted to 5×10^8 colony forming units per ml as described in detail elsewhere (9).

Opsonins. Pooled fresh frozen human serum (PHS) was diluted in Hanks BBS to the desired concentration. In some samples complement was inactivated by heating the samples at 56 C for 30 minutes (IS).

Phagocytosis assay. The assay and methods for calculation of leukocyte associated bacteria, live intracellular bacteria and attached non-ingested bacteria have been described in detail elsewhere (9). Briefly mixtures of leukocyte suspension opsonin and bacteria were tumbled gently in a rotating rack. At different time intervals, duplicate 100 μl samples were drawn from the test mixtures, washed three times in PBS and resuspended in scintillation liquid. The number of leukocyte-associated bacteria was expressed as a percentage of the total number of bacteria (ingested and extracellular) in the test mixtures at the end of the assay period.

To differentiate between cell associated bacteria that were ingested and those bacteria that were attached to the leukocytes, some samples were incubated in 1 μg/ml lysostaphin at 37 C for 30 minutes (9). This enzyme is known to lyse staphylococcal cell walls and does not enter leukocytes. Therefore it will remove attached non-ingested leukocyte-associated bacteria. The difference between per cent uptake calculated from the samples placed in PBS and the samples placed in PBS containing lysostaphin was considered to represent the attached population. The number of live leukocyte-associated bacteria was determined by the pour plate technique.

RESULTS

Increasing the bacteria to neutrophil ratio from 1:1 to 20:1 did not influence the proportion of phagocytized bacteria (Fig 1). At higher ratios the proportion of phagocytized bacteria declined somewhat. Similar results were obtained for varying serum dilutions (2-20 per cent) and for the different bacterial species tested (group B streptococci, *S. aureus*, *S. epidermidis* and *E. coli*), i.e. the varying capacity of these organisms to activate the alternative pathway did not influence the results.

There was no phagocytosis of *E. coli* or of streptococci in heat inactivated serum, suggesting that complement was essential for opsonization of these bacterial species. However addition of heat inactivated serum to diluted fresh serum enhanced opsonization, thus indicating that heat-stable factors played a role in opsonization and that the concentration of heat-stable opsonins was lower than that of heat labile factors (Fig 2).

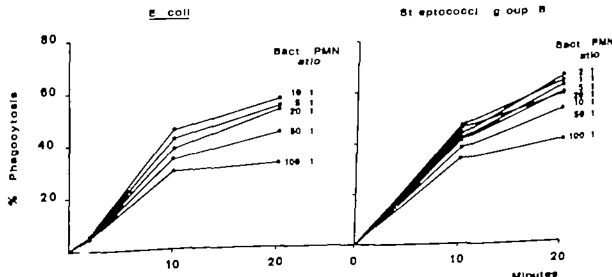


Fig 1. Effect of varying bacteria to PMN ratios on the ingestion of group B streptococci and *E. coli* opsonized in 8 per cent human serum. Figure shows average of three experiments.

Kempe foundation, Sweden and the *Swedish Medical Research Council* (B 77 19x-04990-01)
J V was supported by the *Netherlands Organization for the Advancement of Pure Research* (ZWO)

REFERENCES

1 *Björkstén, B. & Quie P G.:* Abnormalities of phagocyte function. I *Thompson E.* (Ed.): Advances in Immunology Churchill Livingstone, Edinburgh In press, 1977
2. *Clawson, C C. & Repin J E.* Quantitation of maximal bactericidal capability in human neutrophils J Lab. Clin. Med. *88* 316–327 1976
3. *Craig, C P & Suter E.* Extracellular factors influencing staphylocidal capacity of human polymorphonuclears. J Immunol. 97 287–296 1966.
4. *Madsen O* On the relation between alexin and opsonin. Munksgaard, Copenhagen, 1976.
5. *Peterson P K., Verhoef J Sabath, L. D & Quie P G* Extracellular and bacterial factors killing by human polymorphonuclear leukocytes. Infect. Immun. *14* 496–501 1976.
6. *Quie P G., White J G Holmes B. & Good R. A* In vitro bactericidal capacity of human polymorphonuclear leukocytes. Diminished activity in chronic granulomatous disease. J Clin. Invest. *46* 668–679 1967
7 *Solberg, C O.:* Enhanced susceptibility to infection new method for the evaluation of neutrophil granulocyte functions. Acta path. microbiol. scand. Sect. B, *80* 10–18, 1972.
8. *Tan J S Watanakunakorn C & Phair J P.* A modified assay of neutrophil function Use of lysostaphin to differentiate defective phagocytosis from impaired intracellular killing. J Lab. Clin. Med. *78* 316–322, 1971
9 *Verhoef J Peterson, P K., Quie P G & Sabath L. D* Kinetics of staphylococcal opsonization, attachment, ingestion and killing by human polymorphonuclear leukocytes A quantitative assay using ³H-thymidine labelled bacteria. J Immunol. Methods *14* 303–311 1977

vated serum which in itself had no opsonic activity for the two bacterial species (Fig. 2) These findings, and the fact that opsonic activity in any of the tested serum dilutions did not vary when the bacteria to PMN ratios were changed between 1 1 and 20 1 would suggest that there is a threshold for complement activation and when this is reached there is an amplification of opsonization. Similar results were found when we tested a strain with the capacity to activate complement via the alternative pathway (*E coli*) and a strain lacking this capacity (group B streptococci) Apparently the presence of heat-stabile factors enhanced *E coli* opsonization via the classic pathway This pathway is more rapidly activated than the alternative pathway (9)

The limiting factor in phagocytosis was studied by separation of the various steps in the phagocytic process, i.e. attachment, ingestion and killing Using lysostaphin, attachment and ingestion of *S aureus* could be separated. Lysostaphin rapidly lyses *S aureus* but it cannot penetrate into PMNs (8) Thus, by incubating a sample containing PMNs and bacteria with lysostaphin the extracellular but not the intracellular bacteria were lysed. Fig 3 shows the results of experiments in which duplicate samples were suspended in PBS or PBS containing lysostaphin. If all bacteria that were cell associated were ingested, the counts would be expected to be identical in the samples, and consequently any differences between them represent bacteria attached to the PMNs but not ingested by them As shown in Fig 3 the proportion of cell associated non ingested bacteria remained constant when the bacteria to PMN ratio was increased, indicating that bacteria that are attached to the cells are ingested at a constant rate independent of the number of bacteria that are attached. Thus, the limiting factor for phagocytosis appears to be the rate of attachment, presumably reflecting the availability of receptor sites. These experiments were only done with *S aureus* However since the results were similar for the different bacterial species in the other experiments, we have assumed that the data presented in Fig 3 are valid also for *E coli* streptococci and *S epidermidis*

By comparing the proportion of cell-associated live bacteria using the pour-plate technique with the total proportion of cell-associated bacteria as measured by the radioassay we were able to evaluate the proportion of bacteria that were cell-associated and still alive at different times. As shown in Fig 4 the proportions of ingested bacteria that were still alive at 10 and 20 minutes were similar and did not vary with the actual number of ingested bacteria, thus indicating that intracellular killing is not a limiting factor in the phagocytic process. Results similar to these have been reported previously for staphylococci (3 5) These results, however are in contrast to the recent report by *Clawson & Repine* (2) who using very high bacteria to PMN ratios over 100 1 found that killing was a limiting factor in the phagocytic process. They suggested that high bacteria to PMN ratios can be employed as a "stress-test for phagocytic function. However such very high ratios are probably not encountered *in vivo* and the clinical importance of minor abnormalities in phagocytosis demonstrated by this test method is questionable. The reason for the discrepancy between their results and ours might reflect differences in bacteria to PMN ratios and in the sensitivity of the methods used.

The Maaloe method (4) is the most commonly used technique for evaluating phagocytosis. However the validity of measuring killing as an endpoint of phagocytosis has been questioned. This study shows that intracellular killing parallels phagocytosis over a wide range of bacteria to PMN ratios, and that test results obtained using various numbers of bacteria may be comparable The limiting factor in phagocytosis of opsonized bacteria appears to be attachment of the bacteria to the phagocytes and when this occurs, ingestion and subsequent killing are rapid for the four bacteria species we have studied. Thus under *in vivo* conditions, opsonization and attachment probably are the most critical determinants of adequate phagocyte function.

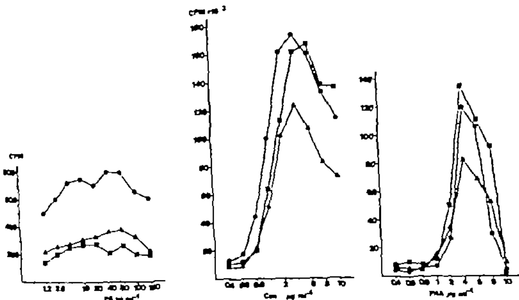


Fig. 1. Response of normal rat lymphoid cells to different concentrations of LPS, ConA, and PHA.
●———● spleen ▲———▲ lymph node. ■———■ blood.

Immunization in vivo was performed by subcutaneous injection into the hind footpads. 1 day 0 1 2 × 10⁷ nucleated F1 spleen cells were given per foot to parental strain rats of either the Hooded or Fischer type. The popliteal lymph node was removed and processed for tissue culture on different days during the first two weeks.

Mixed lymphocyte cultures (MLC) were prepared and the ¹⁴C uptake determined essentially as described by one of us earlier (4).

Mitogens used were PHA P (Wellcome), ConA (Pharmacia) and LPS (Difco).

The concentrations used are indicated under "Results".

Macrophages were removed from single cell suspension combining adherence and phagocytosis by incubating cells with 10 mg carbonyliron per ml for 45 min at 37 C in Falcon flasks and removal by magnetism in three passages. Latex phagocytosis and acridine orange staining of cytoplasmic granules were used as markers.

Fc receptor carrying cells were identified and depleted following EA-rosette formation with sheep red blood cells (SRBC) sensitized by subagglutinating dose of rabbit IgG antibody against SRBC*. Conditions used for rosette formation were incubation of EA and lymphoid cells at ratio of about 50:1 for 30 min at 37 C, followed by centrifugation at 200*G* for 5 min and gentle resuspension. Depletion of EA binding cells was performed after addition of 25 per cent FCS to the rosette mixture. The mixture was layered on Ficoll-Isopaque (specific gravity 1.090) and centrifuged for 30 min at 700*G*. The interphase cells were pipetted off and washed twice.

C3 receptor carrying cells were identified and/or removed following EAC-rosetting with SRBC coated with rabbit IgM anti-SRBC antibodies and C5 deficient mouse serum (obtained from AKR mice). EACI-rosettes, using heat inactivated AKR serum (I) as complement source, were used as controls and resulted in less than 1 per cent rosette forming lymphocytes when tested.

Column fractionation of rat lymphoid cells was carried out by passing the suspension, which was washed three times in RPMI 1640 with FCS, on pretreated Degalan bead columns. Washed Degalan beads were incubated for 1 h at 45 C and overnight at 4 C with rabbit anti-rat immunoglobulin (Ig) purified by elution from an affinity chromatography on Sepharose 4 B coupled with rat Ig.

Suspensions were passed at room temperature at flow rate of about 1 ml per minute. Ten ml of packed Degalan was loaded with around 5 × 10⁷ lymphocytes in 10 ml.

Surface Ig positive rat cells were identified by indirect immunofluorescence according to standard methods.

---

* Kindly donated by *B. Rubin*, Copenhagen.

Acta path. microbiol scand Sect C, 85 350 358, 1977

LOCAL LYMPH NODE RESPONSE IN RATS TO ALLOGENEIC STIMULUS ANALYSIS OF THE *IN VITRO* RESPONDING CELLS

IVER HERON [a]) and HENNING MEYER [b])

a) The Institute of Medical Microbiology Aarhus Universitet, Aarhus, Denmark, and
b) Blood Grouping Laboratories and Institute of Pathology Aalborg, Denmark

Heron, I & Meyer H. Local lymph node response in rats to allogeneic stimulus Analysis of the *in vitro* responding cells. Acta path. microbiol. scand. Sect. C, 85 350–358, 1977

Parental strain rats were immunized in the footpads by F1 hybrid spleen cells. The draining lymph nodes were removed during the first 10 days after immunization and tested for mitogen responsiveness. Cell fractionation was performed by removal of macrophages, surface Ig positive and F(c) and C3-receptor carrying lymphocytes. The depleted suspensions were tested in MLC to test the effect of lymphocyte subpopulations on the accelerated MLC response profile exhibited by the immune lymph node cells. Overall increase in mitogen stimulation was observed but stimulation indices were slightly reduced as compared to non-immunized cells. Removal of phagocytic cells gave some increase in MLC response which was most pronounced when responder cells were tested during the first days after immunization. Depletion for non-T lymphocytes resulted in lowering of non-stimulated responder cell ^{14}C uptake, whereas the specific accelerated MLC profile was unaltered or tended to improve acceleration. It is concluded from indirect evidence that T lymphocytes are the cell type responsible for the altered MLC kinetics, but that macrophages and non-T cells modify the response

Key words Lymph node response allogeneic stimulus cell analysis *in vitro*

I Heron, The Institute of Medical Microbiology Aarhus University Aarhus Denmark.

Received 11.iii.77 Accepted 29 iv 77

Accelerated proliferation of lymphocytes *in vitro* responding against the specific immunizing alloantigens is characteristic in the early period following sensitization with allogeneic cells (1 2 6, 7) This *in vitro* response profile has received the designation "early peak and is seen most markedly when regional lymph node cells are used as responders from animals 5–10 days following subcutaneous immunization The work described in the present paper was undertaken to answer the questions 1) what are the functional characteristics of the cells participating in the early peak" response, as measured by mitogen stimulation *in vitro* and 2) which population of cells is responsible for the accelerated MLC. The experiments were performed in an inbred rat model used previously in studies of the host versus graft reaction (5)

MATERIAL AND METHODS

Rat strains. The animals used were inbred rats of the Hooded Lister strain carrying the AgB[3] allel, Fischer (AgB[1]) and Brown Norway (AgB) and their F1 hybrids.

summarizes such experiments, and shows that increased thymidine uptake in responses against all three mitogens was the major alteration. The PHA responses were found to be increased from 125–153 per cent of normal ConA responses to 134–154 per cent, and LPS responses to 159–160 per cent of the values given by non-immune cells tested in the same experiments. Similar overall increment increases in mitogen responsiveness were found when the regional lymph nodes were tested on days 1–4 after immunization, while rats tested after four weeks had returned to normal response levels. Non-stimulated cultures from recently immunized regional nodes gave almost twice as high counts as controls (Fig. 2 B). If stimulation indices (CPM with mitogen/CPM of non-stimulated) were compared, immunized regional lymph nodes gave the overall decreased mitogen response shown in the column of Fig. 2 A. Distant lymph node lymphocytes gave normal mitogen responses, whereas spleen cells from immunized animals expressed selective increase in LPS response, both absolute and in stimulation index. Lymphoid cells from the blood showed increased conA responses at the lower concentrations and decreased LPS response.

MLC. Table 1 shows MLC results obtain-

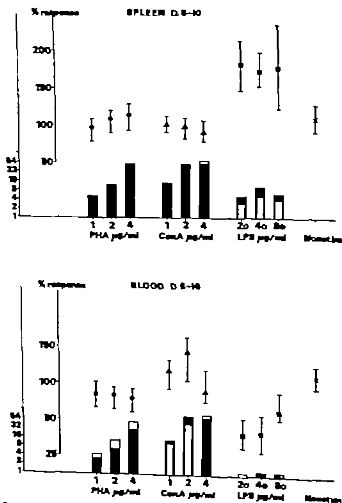


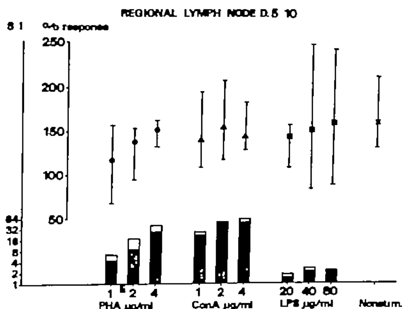
Fig. 2 B

RESULTS

Mitogens Titration of the mitogens was performed initially using recipient strain rats as responders. Fig. 1 shows the results of one such experiment, and the optimal concentrations for use in further culture tests were determined from these results.

One, 2 and 4 μg PHA/ml, 1 2 and 4 μg ConA/ml and 20 40 and 80 μg LPS/ml were chosen. The kinetics were likewise investigated and the optimal day of assaying ^{14}C thymidine incorporation at these mitogen concentrations was determined.

Rats immunized in the footpad were killed at different intervals during the first week, and cells from local lymph node, distant lymph nodes, blood and spleen were tested for mitogen responsiveness. In a few experiments, the total mitogen titrations and culture kinetics were performed on these immune cell suspensions (not illustrated). No surprising shifts in dose response and kinetics were found, and subsequent experiments were performed with a fixed day of culture harvest (after 72 hours) and with a narrow range of mitogen concentrations. Fig. 2A



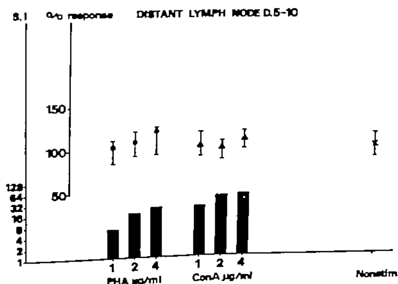


Fig. 2 A

Fig. 2 A B Summary of experiments testing mitogen responsiveness of lymphoid cells from immunized rats.

The response of normal non-immune lymphocytes to certain concentration of mitogen in the single experiment is defined as 100. The relative response (points on the figure) of the immune cells to the same concentration of the mitogen is calculated. The ranges (bars) are shown for each point. The columns represent stimulation indices (SI). The filled parts of the columns indicate SI of immune cells, the top of the open columns SI of non-immune cells.

TABLE 2 *MLC and Mitogen Response with (+) and without (—) Macrophages. Responder Cells are Regional Lymph Node Cells from Rat Immunized in Footpads One or Two Days before Testing in MLC. Median CPM of Triplicate Determinations are Shown*

Responder		MLC Stimulator						Mitogen		
		Specific			Third party					
		2d	3d	4d	2d	3d	4d	PHA 2 μg/ml	ConA 2 μg/ml	LPS 40 μg/ml
Immune day 1	+		5329	35256	–	1869	9668	29990	112466	350
	—	–	9087	66068	–	2841	37410	39586	134501	762
Immune day 1	+	4199	20552	69577	4311	13736	51322	340410	55918	2645
	—	7525	47310	92307	8314	40477	73751	74423	121849	9519
Immune day 2	+	1448	–	15465	1377	–	16181	17630	–	987
	—	14209	–	40362	13173	–	38101	43175	–	7489
Immune day 2	+	462	1800	12918	343	2039	9938	–	122635	1345
	—	806	7448	34223	705	3857	24908	–	166649	1455

TABLE 1 *Impact of Adherent and Phagocytic Cells on MLC Response of Immunized Regional Lymph Node Lymphocytes 5–7 Days after Immunization*

Responder		Specific			Stimulator Third party			Medium		
		2d a)	3d	4d	2d	3d	4d	2d	3d	4d
Exp 1 Normal	+	2932	29436	71482	2011	18483	64157	472	979	1716
	—	1787	23574	69664	1355	21950	74988	328	762	450
Immune	+	9025	62645	45686	2650	13477	51376	922	2315	2523
	—	7826	66124	50767	1976	12356	58576	170	852	708
Exp 2 Normal	+	6598	48189	63875	4911	47988	64438	1504	1715	2253
	—	9761	64905	68797	9530	64582	74150	1550	3027	3534
Immune	+	14924	85220	50158	3378	39404	86578	324	1761	3003
	—	31475	108686	62520	3988	74738	95693	881	1644	1811
Exp 3 Normal	+	2402	18160	60637	1251	7691	45323	488	745	1526
	—	3740	19380	73050	2372	23161	87337	581	3109	3120
Immune	+	11106	64241	33517	1326	10903	55099	473	1641	2488
	—	21826	64476	21322	3305	24744	63363	488	2320	3258
Exp 4 Normal	+	3811	13636	52724	4327	16002	64312	362	896	884
	—	3751	24036	39913	4706	23568	69872	528	890	869
Immune	+	6337	40850	31205	927	8865	47548	624	1923	1289
	—	4809	52874	29130	1111	11379	46979	506	1325	1872

a) *2d, 3d and 4d represent the day of harvest of the culture. The numbers in the table are median CPM of* triplicate cultures. Variation between triplicate cultures seldom exceeded 15 per cent. MLC with (+) and without (—) macrophages among responder cells.

ed with local nodes as responder cells with and without phagocytic cells. The content of macrophages was reduced from an average of 9.8 per cent before to 1.3 per cent after removal by magnetism. Both specific immunizing F1 cells and third party lymphocytes were used as stimulators. It is obvious that macrophages were not responsible or necessary *for the specific accelerated* MLC re*sponse because their removal did not alter* the kinetics of MLC. On the contrary as can be seen in the table they tended to exert a non-specific suppressive effect on MLC ($p < 0.01$ Wilcoxon's test for matched pairs). This is also true for the control cells that responded and was found to be particularly pronounced during the first days after immunization (Table 2). There was no "early peak" MLC at this phase but the MLC response was augmented following depletion of responder cells for macrophages. Similarly an increased response to mitogen was exerted by these responding lymphocytes.

The MLC response following immunization was tested in regional lymph node lymphocytes depleted by rosette techniques for different lymphocyte subpopulations in four experiments. Table 3 shows a typical experiment. *Regional lymph node cells removed from rats five days after immunization were* depleted of EA and EAC rosette forming cells and tested in MLC against the specific immunizing F1 strain and against an allogeneic control (third party F1). Immune cells not depleted and normal non immune lymph node cells before and after EA and EACI removal served as control responders. Fig. 3 shows the outcome *of two experiments* performed *using column fractionation of the preimmunized responder cells*. Though only *partially purified* the results of Table 3 and Fig. 3 indicate clearly that depletion of the

TABLE 2 MLC and Mitogen Response with (+) and without (—) Macrophages. Responder Cells are Regional Lymph Node Cells from Rats Immunized in Footpads One or Two Days before Testing in MLC. Median CPM of Triplicate Determinations are Shown

Responder		MLC Stimulator						Mitogen		
		Specific			Third party					
		2d	3d	4d	2d	3d	4d	PHA 2 μg/ml	ConA 2 μg/ml	LPS 40 μg/ml
Immune day 1	+	–	5329	35256	–	1069	9668	29990	112466	350
	—	–	9087	66068	–	2841	37410	39586	134501	762
Immune day 1	+	4199	20552	69577	4311	13736	51322	340410	55918	2645
	—	7525	47310	92307	8314	40477	73751	74423	121849	9519
Immune day 2	+	1448	–	15465	1377	–	16181	17630	–	987
	—	14209	–	40362	13173	–	38101	43175	–	7489
Immune day 2	+	462	1800	12918	343	2039	9958	–	122655	1345
	—	886	7448	34223	705	5857	24908	–	166649	1455

TABLE 3 *One of Four Experiments Using Rosette Methods for Depletion Enrichment of Responding Cells before MLC CPM of Cultures Harvested on Three Successive Days are Tabulated*

Responding lymph node cells	Lymphocyte markers, per cent			Stimulator: Specific			Third party			Medium		
	Ig	EA	EAC	2d	3d	4d	2d	3d	4d	2d	3d	4d
Normal	23	21	24	196	1508	4512	?90	1683	6803	31	62	35
Normal EA depleted	6	5	7	334	2489	6812	489	4411	9493	41	45	70
Regional	42	46	33	3035	5557	1987	670	3393	9062	130	198	153
Regional EA depleted	11	9	15	4308	5766	1971	1198	4634	10008	39	69	87
Regional EAC depleted	7	6	4	6070	4987	1603	2391	7934	11090	75	83	103
Regional EACI depleted	33	39	30	3501	3768	2091	81?	3739	9676	60	72	48

responder cells for F(c) receptor carrying cells and for Ig positive cells did not remove the accelerated specific response profile. Augmented and increased acceleration of the early peak response was observed together with relative T cell enrichment of the responding population. Depletion for T cells by rosetting could not be carried out because rat lymphocytes do not form T rosettes (3) but killing of T cells by preincubation with a rabbit anti rat brain serum + complement resulted in lympholysis of almost 70 per cent of responder lymph node lymphocytes and total drop out of the MLC response.

An observation that also emerged from the cell separation experiments (Table 3 and Fig 1) followed by MLC was that the increased background found in non stimulated pre immunized responder cell cultures was decreased together with removal of non T cell. This removal of "background" was observed without any lowering of the mixed culture responses.

DISCUSSION

To our knowledge the cells involved in "immunized" MLC response following *in vivo* sensitization have not been characterized previously. The present results show clearly that Ig positive cells (B cells) and F(c) receptor carrying cells are not the responder cells which cause the early peak. The F(c) cells are mostly B cells, but a subpopulation of T cells might also belong in this category as described in the mouse (8). This indicates indirectly that a non-F(c) carrying T cell is the responder.

However the cell population with which these T cells are mixed in the draining node after immunization modifies the response in at least two ways. One is a suppressor effect exerted by phagocytic cells; this was demonstrated most easily in MLC and mitogen responsiveness early after immunization.

The other modifying cell is the B cell (Ig and F(c) and C3 receptor carrying) that causes increased thymidine uptake in non-stimulated responder cell cultures. This last finding may suggest some doubt as to the validity of using stimulation indices in judging MLC response levels, at least in situations where cell suspensions from immunized animals are assessed.

The results of the mitogen stimulation indicate that all subpopulations responding to the different mitogens seem to be represented functionally in almost normal proportions in the post immunization period, with no dramatic drop out or shift in sensitivity to different concentration of mitogen. The overall increase in response might be explained by preculture activation. This point of view is supported by an increase in ^{14}C thymidine uptake also by non stimulated cells. The only marked difference observed was the LPS responses of spleen cells that were found to be augmented selectively.

MLC

EXP 1 EXP 2

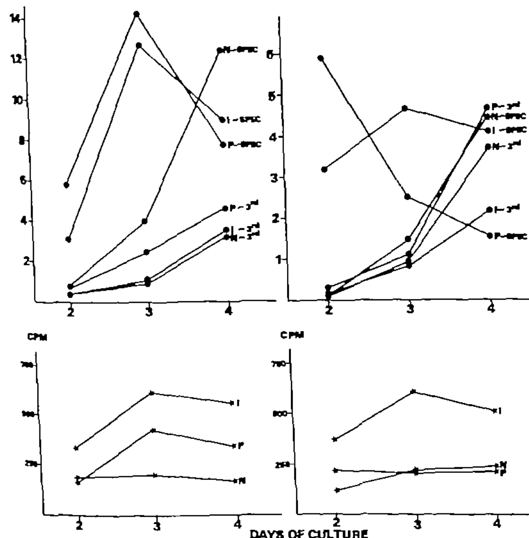


Fig 3 MLC response profiles of immunized lymph node lymphocytes depleted for B cells by passage through an anti-Ig column

N normal non-immune lymph node.

I = lymph node from immunized rats before column passage.

P = column passed cells from immunized rats.

Spec specific stimulator lymphocytes syngene to the F1 cells used for immunization.

3rd = third party stimulator lymphocytes

●——● MLC responses *——* non-stimulated responder cells

Lymphocyte markers (%)

EXP 1 *Normal* Ig 19 6, EA 23 *Immune* Ig 37 EA 34 *Passed* Ig 10 EA 7

EXP 2 *Normal* Ig 26 EA 29 *Immune* Ig 39 EA 42 *Passed* Ig 3.6 EA 8.7

In conclusion lymphoid cells in the draining node following allogeneic stimulation *in vivo* respond in culture to B and T cell mitogens without selective deficiency or augmentation The accelerated MLC response exhibited by these cells when stimulated with the immunizing antigens is dependent on F(c) and Ig negative cells presumably T cells.

Supported by the *Danish Medical Research Council* and *North Jutland Medical Research Foundation*

REFERENCES

1. *Adler W H Takiguchi T Marsh B & Smith R T* Cellular recognition by mouse lymphocytes in vitro J Immunol. *105* 984–1000 1970
2. *Bondevik H & Thorsby E.* The human mixed lymphocyte culture interaction after in vivo alloimmunization. Cell. Immunol. *11* 409–418 1974
3. *Bragenza C M Stathopoulus G Davies A. J S Elliott V & Kerbel R. S* Lymphocyte erytrocyte rosettes as indicators of the heterogeneity of lymphocytes in a variety of mammalian species. Cell. *4* 103–106, 1975
4. *Heron I & Sorensen S F* Role of blocking serum factors in organ grafted rats. Acta path. microbiol. scand. Sect. B *81* 606–614 1973.
5. *Meyer H & Heron I* Regional lymph node response in rats to injection of semiallogeneic lymphocytes Acta path microbiol. scand. Sect. B *81* 724–730 1973
6. *Lerolainen M P., Häyry P & Defendi V* Effect of presensitization on the mixed lymphocyte reaction of rat spleen cell cultures. Transplantation *8* 179–188 1969
7. *Wilson D B & Nowel, P C* Quantitative studies on the mixed lymphocyte interaction in rats. J Exp Med *133* 442–454 1971
8. *Yoshida T O & Andersson B* Evidence for a receptor recognizing antigen complexed immunoglobulin on the surface of activated thymus lymphocytes. Scand. J Immunol. *1* 401–412 1972

Acta path. microbiol scand Sect. C, 85 359-366, 1977

BINDING OF AGGREGATED IgG TO NEPHRITOGENIC TYPE 12 STREPTOCOCCI INFLUENCE OF SERUM C1 AND C4

POUL CHRISTENSEN ANDERS G SJOHOLM and STIG E. HOLM

The Department of Medical Microbiology University of Lund and
The Department of Clinical Bacteriology University of Umeå, Sweden

Christensen, P., Sjöholm, A. G & Holm, S. E. Binding of aggregated IgG to nephritogenic type 12 streptococci Influence of serum, C1 and C4 Acta path microbiol. scand. Sect. C, 85 359-366, 1977

The uptake of aggregated IgG by type 12, M protein positive (M + ve) streptococci was high in the presence of fresh serum, while the uptake by type 12, M — ve type 1 and type 2, M + ve or M — ve streptococci was inhibited. Serum heated to 56 C for 30 min inhibited the uptake of aggregated IgG by all strains tested Purified C1q or macromolecular C1 added to heat-treated serum restituted the uptake of aggregated IgG. It was shown that C1 and C4 in fresh serum influenced the uptake of aggregated IgG by streptococci, resulting in the distinct reaction patterns observed

Key words Streptococci nephritogenicity complement components.

Poul Christensen, Department of Medical Microbiology Sölvegatan 23 S-223 62 Lund, Sweden.

Received 4.iv 77 Accepted 25.iv 77

The association between infection with group A streptococci of certain M types and acute poststreptococcal glomerulonephritis was originally demonstrated by *Rammelkamp et al.* (25 26) and is well established (5 24). Nephritogenic streptococci have not been found to differ from other streptococci with regard to physical or various biological properties (23) Recently *Lange et al.* (12) described a hitherto unknown antigen common to several nephritogenic strains irrespective of M or T type

Many streptococci are capable of binding native human IgG and immune complexes by streptococcal Fc receptors (2, 3 4) These studies suggested that Fc receptor activity in vitro does not distinguish between potentially nephritogenic strains such as M protein positive (M+ve) type 12 streptococci (26) and other streptococci.

The present investigation concerned the interaction of streptococci with ^{125}I labelled aggregated IgG in the presence of serum It was found that fresh serum gave high uptake of aggregated IgG by M+ve type 12 but not by M—ve type 12 streptococci or by M+ve and M—ve variants of two other types tested. Also, it was shown that early acting components of the classical complement pathway were involved in the uptake of aggregated IgG by streptococci in the presence of serum.

MATERIALS AND METHODS

Streptococcal Strains

The following group A streptococci, kindly supplied by the Central Public Health Laboratories, London, were studied: type 1 M+ (S198);

type 2, M+ve and M—ve (S F.59) and type 12, M+ve and M—ve (1130) furthermore, a M—ve type 1 strain (S F 130) kindly supplied by the State Serum Institute, Copenhagen, was used Unless otherwise stated, the streptococci were grown in Todd Hewitt broth (Difco Laboratories Inc., Detroit, Michigan USA) containing 0 02 M Tris buffer pH 8.0 the final pH of this medium after incubation overnight with the streptococci was never below 7 0 A standard suspension of each strain corresponding to about 2.5×10^{10} bacteria/ml was freshly prepared for every experiment, as described previously (3) with the exception that the streptococci were finally suspended in 0.9 per cent saline

Normal and Pathological Sera

Blood specimens were allowed to clot at room temperature. After centrifugation the sera were divided into aliquots and frozen to —80 C The samples were thawed briefly prior to analysis. Sera from 11 apparently healthy donors, 7 patients with hereditary angio-edema, 1 patient with homozygous C4 deficiency and 2 patients with chronic urticaria were used in the investigation Sera were also used after heating at 56 C for 30 min.

Determination of Complement Components

The electroimmunoassay (17) was used for quantitation of C1q, C1r C1s, C4 C3 and CI inactivator (28 29) CI inactivator (CI IA) was determined also by the esterolytic assay of *Levy & Lepow (18)* as modified by *Laurell et al* (14) Hemolytic assay of C1 C4 and C2 were performed mainly according to procedures published by *Rapp & Borsos* (27)

Inulin Treatment of Serum

Inulin treatment of serum was carried out as described by *Götze & Müller Eberhard* (11) Conversion of Factor B was assessed by immunoelectrophoresis, while crossed immunoelectrophoresis (8) was used for the demonstration of C3 conversion products.

Chelation of Serum

In some experiments, normal serum was chelated with 0 01 M EDTA or Mg^{++}EGTA as described by *Forsgren et al* (7)

Purification of CI C1q C2 C4 and CI IA

C1q was prepared according to *Yonemasu & Stroud* (31) Macromolecular CI was purified from neutral euglobulin by gel filtration on Sepharose 6 B CL (Pharmacia Fine Chemicals AB Uppsala Sweden) in acetate buffer pH 5.5 0.2 M NaCl, 5 mM $CaCl_2$ (9)

Functionally purified C4 and C2 were obtained as described earlier (15) A concentrate of CI IA (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service Amsterdam) was chromatographed twice on DE 32 cellulose at pH 7.5 and further purified by gel filtration on Sephadex G 200

Preparation and Radioiodination of Human Aggregated IgG

Commercial IgG (AB Kabi, Stockholm, Sweden) was further purified by chromatography on DEAE cellulose and gel filtration on Sephadex G 200. Heat aggregation was carried out at 63 C for 10 min. Aggregates were isolated by gel filtration on Sepharose 6 B (Pharmacia Fine Chemicals AB, Uppsala, Sweden)

The heat aggregated IgG was labelled with ^{125}I according to *McConahey & Dixon* (21) The protein was measured with a modification of *Fohn's* method (19)

Test for uptake of ^{125}I Labelled Aggregated IgG by the Streptococci

The experiments were performed in plastic tubes (70 × 11 mm, A/S NUNC, Roskilde, Denmark) All dilutions were performed in 0.9 per cent saline. The following procedure was used to test the uptake of ^{125}I-labelled aggregated IgG by the streptococci

(1) incubation of 1 µg IgG aggregates (50 µl) with serum at 37 C for 30 min in some experiments, solutions of complement factors were added to the serum before aggregated IgG The amounts of serum were varied in individual experiments
(2) addition of streptococci (200 µl standard suspension) and further incubation at 22 C for 30 min
(3) addition of 2 ml 0.9 per cent saline and separation of bacteria by centrifugation at 3000 g for 45 min
and
(4) measurement of the radioactivity in the pellet by counting in a gamma scintillation counter (Selektronik, Hoersholm, Denmark)

The adherence of IgG aggregates to the plastic tubes and the amount of aggregates sedimented by centrifugation without reacting with the streptococci were determined as follow 1 µg aggregated IgG (50 µl) was added to 10 50 or 100 µl serum and the tubes incubated at 37 C for 30 min 200 µl of a 1 per cent suspension of human red cells from the same individual as the serum were added in substitution for bacterial particles. The radioactivity in the pellet was determined as described above The radioactivity in the red blood cell sediment did not vary with the amounts of serum and was found to be 22 per cent of the radioactivity added with the use of heat inactivated serum and 18 per cent with fresh serum. These corrections were used in subsequent experiments

RESULTS

Uptake of ¹²⁵I Labelled IgG Aggregates in the Presence of Serum by M+ve and M−ve Type 12 Streptococci

Influence of normal serum M+ve as well as M−ve type 12 streptococci react with native human IgG via receptors for IgG Fc (2) In the absence of serum the uptake of aggregated IgG by the M+ve and M−ve variants were approximately 50 per cent of 1 μg added. We expected that preincubation of aggregated IgG with serum should result in diminished uptake of aggregated IgG by streptococcal Fc receptors, due to competition with unlabelled IgG in serum. As predicted preincubation of IgG aggregates with increasing serum volumes, from 0.1 to 10 μl gave diminished uptake of aggregated IgG by both strains. However when larger volumes of fresh serum were added (50 to 100 μl) the uptake of IgG aggregates by the M+ve streptococci rose sharply (Fig 1A) In contrast, the uptake of IgG aggregates by M−ve type 12 streptococci was further diminished (Fig. 1B) In the presence of the same amounts of heat treated serum the uptake of aggregated IgG by both variants was almost completely inhibited In all experiments described above fresh or heat treated sera from 11 apparently healthy individuals showed the same patterns as described.

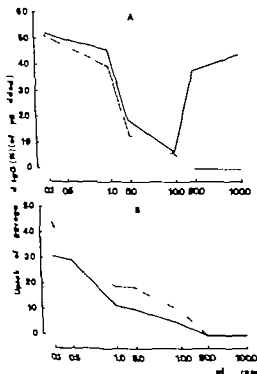


Fig. 1 Uptake of ¹²⁵I-labelled aggregated IgG preincubated in fresh (unbroken line) or heat inactivated (broken line) serum, by streptococci group A, type 12 M+ (Fig. 1A) or M−ve (Fig 1B) as found in typical experiment.
Abscissa: μl serum preincubated with the aggregated IgG (log. scale)
Ordinate: the uptake, expressed in per cent of 1 μg aggregated IgG added.

Determination of optimal test conditions In a series of preliminary experiments the optimal test conditions of the reaction was further defined. Preincubation of labelled IgG aggregates with serum (50 μl) at 37 C was carried out for 1 to 120 min before addition of the streptococcal suspension. Between 15 and 60 min of incubation, the uptake of IgG aggregates in fresh or in heat-treated serum by the streptococci showed minimal variation Therefore, preincubation was carried out for 30 min at 37 C in subsequent experiments.

Next, the influence of incubation time at 22 C of streptococci with the preincubated mixture of IgG aggregates and serum was investigated. Variation of the incubation time from 1 to 120 min showed that the uptake of aggregated IgG by the streptococci decreased after 30 min of incubation. An incubation time of 30 min at 22 C was chosen for streptococci with serum containing the IgG aggregates. Incubation at 37 C or 4 C did not markedly influence the outcome of the analysis.

Incubation of the streptococci with IgG aggregates before serum was added–or with serum before addition of IgG aggregates–did not result in the difference between the reactivities of M+ve and M− ve type 12 streptococci described above.

Thus, the standardized test system used was (1) incubation of IgG aggregates and

serum at 37° C for 30 min and (2) addition of streptococci and further incubation at 22° C for 30 min

The experiments were performed with streptococci grown in media containing 0.02 M Tris buffer at pH 8.0. Streptococci, heat killed at 56° C for 30 min, did not differ in reactivity from live bacteria. Interestingly M+ve type 12 streptococci grown in unbuffered Todd Hewitt broth for 48 hours allowing pH to fall to 5.1–5.2, were capable to bind *native or aggregated IgG* but showed decreased uptake in the presence of fresh serum.

Influence of chelating agents. The uptake of aggregated IgG by M+ve and M—ve type 12 streptococci was measured in repeated experiments after preincubation of IgG aggregates in fresh serum chelated with EDTA or Mg^{++}EGTA. The uptake of aggregated IgG by the M—ve streptococci increased on addition of increasing amounts of EDTA to serum. At a final concentration of 0.01 M EDTA in serum the uptake of aggregates by M—ve streptococci approached that of M+ve type 12 streptococci. Apparently binding of IgG aggregates by the latter strain was not influenced by the presence of EDTA. The addition of EDTA to heat treated serum had no effect. Mg^{++}EGTA in fresh serum influenced the uptake by M—ve and M+ve type 12 streptococci essentially in the same way as did EDTA (Table 1).

Influence of C1q. Since C1q is known to be a heat labile serum component capable of reacting with IgG aggregates in the absence of calcium ions (22) it was thought that high uptake of IgG aggregates by streptococci in the presence of serum might be mediated by C1q. Heat treated serum with various amounts of purified C1q added was incubated with aggregated IgG. The uptake of IgG aggregates by the streptococci in the presence of C1q correlated to the amounts of C1q added. When C1q was added at a final concentration corresponding to normal C1q levels in serum, the uptake of aggregated IgG by M+ve and M—ve streptococci was in repeated experiments the same as in the absence of serum (Table 1). Similar results were obtained with purified macromolecular C1 (C1qrs).

TABLE 1 *The Influence of Some Serum Preparations and of C1q on the Uptake of Aggregated IgG by M+ve and M—ve Type 12 Streptococci*

Serum [a]	Uptake of ^{125}I labelled aggregated IgG (in % of 1 µg added) by streptococci	
	Type 12 M+ve	Type 12 M—ve
Fresh serum	52	0
Chelated serum [b]	52	48
Heat-treated serum	0	0
Heat treated serum + C1q, 25 % [c]	18	16
Heat-treated serum + C1q, 50 %	45	40
Heat treated serum + C1q 100 %	54	54
Inulin-treated serum	52	0
Buffer control	50	50

[a]) 50 µl volumes used.
[b]) EDTA, EGTA or Mg^{++}EGTA 0.01 M with respect to serum.
[c]) Values refer to the concentration of C1q in per cent of the level in normal serum. Substitution of C1q with macromolecular C1 gave essentially the same results as indicated in the table.

Influence of sera with defined complement component deficiences. C1q-deficient serum from two patients with chronic urticaria (16) inhibited the uptake of aggregated IgG by M+ve or M—ve type 12 streptococci, even in the presence of EDTA. In contrast, serum from a patient with homozygous C4 deficiency gave high uptake of aggregated IgG by the M+ve as well as the M—ve variant. The same pattern was observed when sera from patients with hereditary angio-edema were employed provided that C4 and C2 titres were sufficiently low. Restoration to normal levels of C4 in C4-deficient serum by the addition of purified C4 and subsequent incubation with aggregated IgG gave inhibited uptake by M—ve type 12 streptococci, but

did not change the ratio bound by the M+ve *variant*. C4 added to hereditary angio-edema serum reduced the uptake of aggregated IgG by M−ve streptococci. Further inhibition was observed when C2 was also added to such serum. These findings were reproducible with the sera used. The results of typical experiments are summarized in Table 2.

Influence of inulin-treated sera. Normal serum treated with inulin in order to deplete serum of alternate pathway components (7, 11) was incubated with aggregated IgG. The same uptake of IgG aggregates by M+ve and M−ve type 12 streptococci was observed as when untreated normal serum was used (Table 1).

The sera deficient in complement components used in the present investigation showed virtually normal conversion patterns of Factor B and C3 after incubation with inulin.

Analysis of the Uptake of IgG Aggregates by Other Group A Streptococci in the Presence of Serum

Four other strains were tested for uptake of IgG aggregates previously incubated in fresh, fresh chelated (EDTA) or heat inactivated serum (50 µl volumes): the strains were M+ve and M−ve variants of type 1 and 2. No differences between the paired strains were observed with respect to uptake and their reactivity patterns were essentially the same as the M−ve, type 12 streptococcal pattern.

DISCUSSION

The present study showed that appropriate amounts of fresh serum gave high uptake of ^{125}I labelled aggregated IgG by M+ve type 12, group A streptococci, similar to the up-

TABLE 2. *Uptake of Aggregated IgG by M+ve and M−ve Type 12 Streptococci in the Presence of Complement Component Deficient Sera*

Serum)	Complement levels: Electroimmuno assay b)				Hemolytic assay c)			Uptake of ^{125}I-labelled aggregated IgG (in % of 1 µg added) by streptococci	
	C1q	C1	C1r	C3	C1	C4	C2	Type 12, M+ve	Type 12, M−ve
C1q deficient	<5	65	92	51	<5	92	NT d)	0	0
C4 deficient	83	89	100	135	NT	<1	139	53	47
C4 deficient + C4	83	89	100	135	NT	50	139	54	0
HAE) I	NT	NT	NT	72	100	8	99	54	21
HAE II	NT	NT	NT	83	120	<1	<1	54	55
+C1 IA f)	NT	NT	NT	83	120	<1	<1	54	55
+C1 IA + C4	NT	NT	NT	83	120	50	<1	54	27
+C1 IA + C4 + C2	NT	NT	NT	83	120	50	50	54	15
+C1 IA + C2	NT	NT	NT	83	120	<1	50	51	5
Normal serum	NT	NT	NT	120	100	100	100	52	0
Buffer control	–	–	–	–	–	–	–	50	50

) 50 µl volumes used.
b) Values given relative to normal reference.
) Values given relative to the normal serum listed in the Table.
d) Not tested.
) Hereditary angio-edema serum.
f) Added at final concentration of 15 U/ml serum.

take obtained in the absence of serum. In sharp contrast, the uptake of aggregated IgG by other strains (type 12 M—ve type 1 M+ve and M—ve type 2, M+ve and M—ve) was decreased in the presence of fresh serum Serum treated at 56 C for 30 min inhibited the uptake of aggregated IgG by all strains tested (Table 1)

Efforts were made to identify the factors in fresh serum responsible for the high uptake of aggregated IgG by M+ve type 12 streptococci The finding that the activity was heat labile and apparently independent of calcium ions suggested that C1q (22) might be involved in the reaction Indeed, serum deficient in C1q (chronic urticaria) decreased the binding of aggregated IgG by the M+ve type 12 strain (Table 2) On the other hand, chelated serum, or purified C1q added to heat treated serum gave high uptake by M—ve as well as by M+ve type 12 streptococci showing that a requirement for C1q could not explain the different reactivities of the two strains in the presence of fresh serum.

Sera deficient in C4 (homozygous deficiency) or C4 and C2 (hereditary angio-edema) gave high uptake of aggregated IgG by both type 12 variants However C4 added to such sera resulted in decreased binding of IgG aggregates by M—ve type 12 streptococci but not by the M+ve type 12 strain (Table 2) Evidently the different uptake of aggregated IgG by the M+ve type 12 streptococci as compared with the uptake by the other strain was dependent on the presence in serum of at least C1 and C4 The finding that addition of C2 together with C4 to hereditary angio-edema serum, further reduced the binding of IgG aggregates by M—ve type 12 streptococci indicated that factors such as C3 might also participate in the reaction. Results of experiments using inulin treated serum of Mg^{++}EGTA serum did not suggest involvement of alternate pathway activation of complement. The precise mechanism by which early acting components of the classical complement pathway influence the binding of aggregated IgG by streptococci is subject to further investigation.

The question arises as to the role of streptococcal Fc receptors (2) in the test system described. The M+ve and M—ve type 12 variants showed similar Fc receptor activity and the M+ve type 1 streptococci used in the investigation possessed greater Fc receptor activity than the M+ve type 12 strain (4) The observation that M+ve type 12 streptococci cultured in unbuffered media failed to show high uptake of aggregated IgG in the presence of fresh serum could possibly be related to activation of streptococcal proteinase at low pH (6) destroying bacterial structures or serum factors essential for the reaction Thus bacterial factors other than Fc receptors might be involved in complement dependent uptake of aggregated IgG by streptococci.

Acute poststreptococcal nephritis is thought to result from the deposition of complement activating immune complexes in the glomeruli (20) The regular occurrence of hypocomplementemia in these patients (13) further suggests that complement activation plays a role in the pathogenesis of the disease. In this context, the present findings may be of interest, although a causal relationship between nephritogenecity and increased uptake of IgG aggregates in fresh serum by M+ve type 12 streptococci remains speculative.

It is generally agreed that M+ve type 12 streptococci are potentially nephritogenic (10 30) Concerning type M 1 strains, *Rammedkamp & Weaver* (25) failed to observe any instances of nephritis following a large number of infections. There is little epidemiological evidence (1) that pharyngeal infections with type 2 streptococci are associated with glomerulonephritis.

The main point emerging from the present investigation was that early acting components of the classical complement pathway modified the binding of aggregated IgG by streptococci, yielding high uptake by a potentially nephritogenic strain (type 12, M+ve) but not by non nephritogenic streptococ

d tested Preliminary results indicate that high uptake of aggregated IgG in the presence of C1 and C4 in serum is a characteristic of several nephritogenic laboratory strains irrespective of M or T type. Work is in progress to define the bacterial factors involved in the reaction. The possibility is considered that the nephritogenic potential of some streptococci might be explained by properties responsible for the different reaction patterns of nephritogenic and non-nephritogenic streptococci in the test system described.

REFERENCES

1. *Anthony B. F. Yamauchi, T. Pease J. S. Kamon, I. & Chapman S. S.* Classroom outbreak of scarlet fever and acute glomerulonephritis related to type 2 (M 2, T 2) group A *Streptococcus* J. Infect. Dis. *129* 336–340, 1974
2. *Christensen P. J. hansson B. G. & Kronvall, G.* Interaction of streptococci with the Fc fragment of IgG. Acta path. microbiol. scand. Sect. C, *84* 73–76, 1976
3. *Christensen, P. & Oxelius V. A.* Quantitation of the uptake of human IgG by some streptococci groups A, B, C and G. Acta path. microbiol. scand. Sect. B *82* 475–483 1974
4. *Christensen P. Soltess, L. V. & Oxelius V.-A.* Visualization of the binding of IgG myeloma proteins to some group A streptococci. Acta path. microbiol. scand. Sect. C, *84* 203–209 1976.
5. *Dillon, H. C.* Streptococcal infections of the skin and their complications impetigo and nephritis I. *Wannamaker L. W. & Matsen, J. M.* (Eds.) Streptococci and streptococcal diseases. Academic Press, New York and London 1972 pp. 571–580
6. *Elliot S. D.* Streptococcal proteinase. I. *McCarty M.* (Ed.) Streptococcal infections. Columbia University Press. New York 1954 pp. 56–64
7. *Forsgren A. M. Leon R. H. Michael, A. F. & Quie P. G.* Studies of the alternate pathway in chelated serum J. Lab. Clin. Med. *85* 904–912, 1975
8. *Ganrot P. O.* Crossed immunoelectrophoresis Scand. J. clin. lab. Invest. 29 suppl. *129* 39–41 1972.
9. *Gigli, I. & Porter R. R.* Preparation of unactivated human C1 and activation of the C1 subunits. J. Immunol. *116* 1733–1734 1976.
10. *Ginsburg I.* Mechanism of cell and tissue injury induced by group A streptococci relation to poststreptococcal sequelae. J. Infect. Dis. *126* 419–456 1972.
11. *Götze O. & Müller-Eberhard H. J.* The C3 activator system, an alternate pathway of complement activation. J. exp. Med. *134* 90s–108s 1971
12. *Lange K., Ahmed U., Kleinberger H. & Treser G.* A hitherto unknown streptococcal antigen and its probable relation to acute poststreptococcal glomerulonephritis. Clin. Nephrol. *5* 207–215 1976
13. *Lange K., Wasserman E. & Slobody L. B.* The significance of serum complement levels for the diagnosis and prognosis of acute and subacute glomerulonephritis and lupus erythematosus disseminatus. Ann. Intern. Med. *53* 636–646, 1960
14. *Laur II, A. B., Landh, B. & Malmquist J.* Inability of a highly purified streptokinase preparation to inactivate complement in serum. Acta path. microbiol. scand. *64* 318–328 1965
15. *Laurell, A.-B., Mårtensson U. & Sjöholm, A. G.* C1 subcomponent complexes in normal and pathological sera studied by crossed immunoelectrophoresis. Acta path. microbiol. scand. Sect. C, *84* 455–464 1976
16. *Laurell, A.-B., Mårtensson U. & Sjöholm, A. G.* Studies of C1 subcomponents in chronic urticaria and angio-edema. Int. Arch. Allergy *54* 434–442, 1977
17. *Laurell, C.-B.* Electroimmuno assay Scand. J. clin. lab. Invest. 29 suppl. *128* 21–37 1972.
18. *Levy L. R. & Lepow I. H.* Assay and properties of serum inhibitor of C'1 esterase. Proc. Soc. exp. Biol. *101* 608–611 1959
19. *Lowry O. H. Rosebrough, N. J. Farr J. A. & Randall R. J.* Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193* 265–275 1951
20. *Michael A. F. Drummond K. N., Good, R. A. & Vernier R. L.* Acute poststreptococcal glomerulonephritis: immune deposit disease. J. clin. Invest. *45* 237–247 1966
21. *McConahey P. J. & Dixon F. J.* A method of trace iodination of proteins for immunologic studies. Int. Arch. Allergy 29 185–189 1966.
22. *Müller-Eberhard H. J.* Isolation and description of proteins related to the human complement system. Acta Soc. Med. Upsalien. 66 152–170 1961
23. *Noble R. C. & Vosti K. L.* Biologic and immunologic comparison of nephritogenic and non-nephritogenic strains of group A, M-type 12 *Streptococcus*. J. Infect. Dis. *128* 761–768, 1972.
24. *Rammelkamp C. H. J.* Concepts of patho-

genesis of glomerulonephritis derived from studies in man. In: *Uhr J W* (Ed.) The streptococcus, rheumatic fever and glomerulonephritis. The Williams & Wilkins Company Baltimore 1964 pp. 289–300

Rammelkamp C H Jr & Weaver R. S Acute glomerulonephritis significance of variations in incidence of disease J Clin, Invest. *32* 345–350 1953

Rammelkamp C H Jr Weaver R. S & Dingle J H Significance of the epidemiological differences between acute nephritis and rheumatic fever Trans. Ass. Amer Physicians *65* 168–179 1952

Rapp H J & Brorsos T Molecular basis of complement action. Appleton-Century-Crofts, New York 1970

28. *Sjöholm A G* Complement components in normal serum and plasma quantitated by electroimmuno assay Scand. J Immunol. *4* 25–30 1975

29. *Sjöholm A G Mårtensson U & Laurell, A B.* C1r levels in normal sera determined by electroimmuno assay Acta path. microbiol. scand. Sect. C *84* 425–427 1976.

30. *Stollerman G H* Rheumatogenic and nephritogenic streptococci. Circulation *43* 915–921 1971

31. *Yonemasu K & Stroud R M* C1q rapid purification method for preparation of monospecific antisera and for biochemical studies. J Immunol *106* 304–313 1971

Acta path. microbiol. scand. Sect. C, 85 367–372, 1977

ALKALINE PHOSPHATE IN THE DIFFERENTIATION OF GUINEA PIG T LYMPHOCYTES

Esa Soppi, Olli Ruuskanen and Kauko Kouvalainen

Departments of Anatomy Medical Microbiology and Pediatrics, University of Turku, 20520 Turku 52, and Department of Pediatrics, University of Oulu, Finland

Soppi, E. Ruuskanen, O. & Kouvalainen, K. Alkaline phosphatase in the differentiation of guinea pig T lymphocytes. Acta path. microbiol. scand. Sect. C, 85 367–372, 1977

The majority of the guinea pig thymocytes have an intensive alkaline phosphatase (AP) activity whereas less than one per cent of the peripheral lymphocytes are AP positive. The number of peripheral AP positive lymphocytes decreased after thymectomy and after antithymocyte serum treatment, and some of them formed E-rosettes with rabbit red blood cells. The observations support the idea that some of these AP positive peripheral lymphocytes are T cells. It seems evident that the AP positive cortical (immature) thymocytes contain two separate cell lines during the maturation one differentiates into AP negative and the other remains AP positive. This differentiation seems to occur within the thymus.

Key words Alkaline phosphatase guinea pig T lymphocytes differentiation.

E. Soppi, Department of Anatomy University of Turku, SF 20520 Turku 52, Finland.

Received 24 77 Accepted 17 v 77

We have demonstrated previously that guinea pig thymocytes contain an intensive alkaline phosphatase (AP) activity which the majority of the cells lose during differentiation into medullary thymocytes and T cells (12, 20, 22). In the present paper we have studied a very small AP positive subpopulation of peripheral lymphocytes. The observations indicate that some of these cells are T cells and support the two lineage model in thymocyte differentiation (3 8, 9 21).

MATERIALS AND METHODS

Animals

Outbred Dunkin-Hartley female and male guinea pigs were used. The animals were killed at the age of 6–16 weeks.

Cell Suspensions

Cell suspension smears were prepared from blood and lymph. Thymus, mesenteric and cervical lymph nodes, spleen bone marrow and Peyer's patches were gently teased with forceps in saline for the smears. The lymphatic lympocytes were collected from the mesenteric lymphatic duct under spinal anaesthesia (24) according to the method of *Dinen & Adams* (2)

Operative Procedures

For the neonatal thymectomy ether anaesthesia was used. The intrauterine thymectomy was performed under spinal anaesthesia (24) and under strict aseptic conditions, as described by *Merikanto et al.* (13)

Preparation of Antithymocyte Serum (ATS)

The method described by *Polak & Turk* (15) was used. Rabbits were injected twice with 5×10^{8} guinea pig thymocytes. The first time the cells were given together with complete Freund' adju-

vant and injected both sub- and intracutaneously The second cell injection was given only intravenously without adjuvant. The rabbits were bled from the ear vein one week after the last injection. The serum was incubated at 56 C for 30 min to inactivate complement and adsorbed with guinea pig erythrocytes. The erythrocytes were separated from the heparinized guinea pig blood with Plasmagel (3 1) (Yliopiston Apteekki, Helsinki Finland) The serum was sterilized by passage through a 0 45 μm millipore filter before use Normal rabbit serum subjected to the same procedures was used as a control serum. The ATS was tested by studying the effect of one dose (2 ml) on the number of blood lymphocytes, which decreased about 80 per cent in one hour and remained thus for the interval of 24 h studied.

ATS and Cyclophosphamide Treatment

Two ml of ATS or control serum was injected intraperitoneally (i. p.) on six successive days and the animals were killed on the next day One dose of cyclophosphamide 300 mg/kg (Lääke Oy Turku, Finland) was given i p and the animals were killed three days later (25)

Preparation of E rosettes

The E-rosettes were prepared according to the method described by *Stadecker et al.* (23) Erythrocytes were removed from the guinea pig spleen cell suspensions by hypotonic shock 6 ml of distilled cold water was added to the cell suspension (in 2 ml of saline) and shaken for 30 s, and the solution was isotonized by adding 2 ml of 3.5 per cent NaCl The rabbit red blood cells were separated using heparinized Plasmagel. Erythrocytes were washed twice in phosphate buffered saline pH 7 4 and concentrated to 100 × 10^6 cells/ml of veronal saline buffer (VSB) with 1 per cent bovine serum albumin. Equal volumes (100 μl) of erythrocyte suspension and lymphoid cell suspension containing 4 0 × 10^6 cells per ml of VSB were incubated together at 37 C for 30 min The cells were then spun down at 200 g and the pellet was left at 4 C for 60 min The cell suspension was mixed gently with a Pasteur pipette and cell smears were prepared One hundred lymphocytes were counted in order to study the number of rosette-forming cells.

Demonstration of Alkaline Phosphatase

The cell smears were fixed in cold acetone for 5 min and alkaline phosphatase (AP) was demonstrated by the calcium cobalt method with β-glycerophosphate (Merck, Darmstadt, Germany) as substrate (14) An incubation time of 45 min was used throughout the study One to three thousand cells were studied when investigating the number of AP positive cells.

RESULTS

The number of AP positive lymphocytes in different guinea pig lymphoid organs is shown in Table 1 In the thymus they form 96 per cent of the thymic lymphocytes and in the peripheral lymphoid tissue less than one per cent of the lymphocytes. The difference indicates that there is considerable variation in the numbers of the peripheral AP positive lymphocytes. When studied under light microscopy the AP positive cells in the thymus are indistinguishable from those in blood, lymph and other lymphoid tissues (Fig 1)

TABLE 1 *Normal Numbers (Percentage of the Lymphocytes) of AP Positive Lymphocytes in Guinea Pig Lymphoid Tissue*

	% ± SEM	N
Thymus	96.38 ± 0 30	20
Blood	0 26 ± 0 05	20
Lymph nodes	0.89 ± 0 14	20
Spleen	0 21 ± 0 04	20
Bone marrow	0 16 ± 0 04	20
Lymph	0 46 ± 0.17	9
Peyer's patches	0.36 ± 0 18	11

N refers to the number of animals studied.

Thymectomies were performed at two different ages in utero at the gestational age of about 40–50 days (the gestational period in guinea pig is about 65–70 days) and neonatally The in utero and neonatally thymectomized animals were killed at the age of 6–16 weeks. In both groups the thymectomy induced a decrease in the number of AP positive lymphocytes in the lymph nodes (Table 2) This decrease was statistically significant in the animals operated in utero. Thymectomies did not induce changes in the number of splenic AP positive lymphocytes.

Antithymocyte serum (ATS) treatment induced a statistically significant decrease in the number of AP positive lymphocytes, both in the lymph nodes and spleen (Table 3)

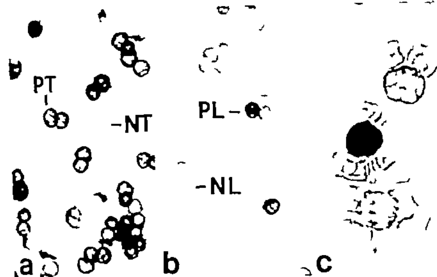


Fig 1) Thymocyte suspension the majority of the cells are AP positive (PT) two AP negative (NT) cells can be seen in the middle of the picture. × 500 b) Lymph node cell suspension the majority of the cells are AP negativ (NL) two AP positiv lymphocytes (PL) can be seen. × 500) An AP positive rosette forming lymph node lymphocyte can be seen in the middle of the picture. × 1500. All cell smears are stained for the demonstration of AP

TABLE 2 *The Effect of Thymectomy on the Number (Percentage of Lymphocytes) of AP Positive Peripheral Lymphocytes*

	Thymectomy					
	Intrauterine	N	Neonatal	N	Control	N
Lymph node	0.29 ± 0.07	10	0.47 ± 0 08	10	0.89 ± 0.14	20
Spleen	0.21 ± 0.08	10	0.27 ± 0 06	10	0.21 ± 0 04	20

Mean ± SEM is given.
N refers to the number of animals studied
When compared with the normal controls, the difference is significant, = P < 0 01 (Student's t-test)

TABLE 3 *The Effect of Antithymocyte Serum (ATS) Treatment on the N mber (Per entage f Lymphocyte) of AP Positive Lymphocytes*

	ATS treated	N	Control	N
Lymph nodes	0 21 ± 0 07*	13	1.03 ± 0 36	6
Spleen	0.10 ± 0.03**	13	0 31 ± 0 04	6

Mean ± SEM is given.
N refers to the number of animals studied.
When compared with the controls, the difference is gnificant, = P < 0 01 ** = P < 0 05 (Student' t-test)

TABLE 4 *Per entage of E-rosette Forming Lymphocyt in Different Lymphoid Tissues and among AP Positive (AP+) Lymphocytes*

	E-rosettes		
	All lymphocytes	AP+ lymphocytes	N
Lymph nodes	38 ± 3	62 ± 4	12
Spleen	25 ± 2	21 ± 4	12
Thymus	82 ± 2	92 ± 2	12

Mean ± SEM is given.
N refers to the number of animals studied.

Furthermore ATS induced a decrease from 38 per cent to 27 per cent in the number of rosette forming lymphocytes in lymph nodes but had no effect on the number of rosette forming lymphocytes in the spleen.

One dose of cyclophosphamide (300 mg/kg) had no effect on the proportional number of AP positive peripheral lymphocytes. Three days after the injection the number of AP positive lymphocytes was 1 00 ± 0 05 per cent in the mesenteric lymph nodes and 0 13 ± 0 05 per cent (N = 4) in the spleen.

Eighty two per cent of thymocytes, 38 per cent of the lymph node lymphocytes and 25 per cent of the splenic lymphocytes formed E-rosettes with rabbit red blood cells (Table 4) The proportional numbers of AP positive rosette-forming lymphocytes (Fig 1 c) varied markedly The majority 92 per cent, of the AP positive thymocytes formed E-rosettes, while the figures in the lymph node and spleen cell smears were 62 and 21 per cent, respectively (Table 4)

DISCUSSION

Although the biological function of AP is unknown (5) the enzyme provides a useful marker for studies on the differentiation and maturation of guinea pig thymocytes and T lymphocytes In this study it was found that at least some of the peribheral AP positive lymphocytes are T cells. Their number became decreased after thymectomy and after ATS treatment. Some of them formed E-rosettes with rabbit red blood cells, which is known to be a sign of T cells in guinea pigs (10 23 27) Furthermore in a previous study (20) is was noted that AP positive lymphocytes appear in the embryonic lymphoid tissues after the thymus has differentiated thus supporting the idea that these cells are thymus derived.

Recently we have demonstrated two subpopulations of guinea pig thymocytes with different maturation stages. One contains strongly AP positive thymocytes which cannot be stimulated by PHA and Con A Their migration properties also differ from mature T cells and they are considered to be immature thymocytes. The other cell population contains weakly AP positive thymocytes which react to PHA and Con A stimulation but have immature homing properties. The weakly AP positive subpopulation of thymocytes is considered to represent an intermediate maturation degree between cortical AP positive and medullary AP negative thymocytes (22) On the basis of this work and our previous studies (18, 22) it seems evident that the AP positive immature thymocytes contain two separate cell lines. During maturation one differentiates into AP negative cell population within the thymus. This possibility is supported by the finding that no significant difference could be observed in the number of AP positive lymphocytes in the thymic arterial and venous blood (19) The other cell population remains AP positive during migration from the thymus into the periphery and forms the small subpopulation of peripheral T cells. However it cannot be ruled out that these peripheral AP positive T cells differentiate further into AP negative cells. This differentiation would in any case be rather slow since thymectomy induced in 6–16 weeks a decrease but not a disappearance of the AP positive cells. In a previous study it was found that some spleen-seeking AP positive thymocytes are able to continue living outside the thymic microenvironment this subpopulation of thymocytes may represent the AP positive mature T cells in the thymus (18)

The different responses of the lymph node and splenic AP positive lymphocytes to ATS, intrauterine and neonatal thymectomies favour the view that heterogeneity also exists among this cell population. The heterogeneity of guinea pig T cells is also supported by the findings of *Andersson et al* (1) who have shown that lymph node and splenic T cells have different electrophoretic mobilities. The different distribution of rat bone marrow lymphocyte antigen within the lymph node and splenic T cells also indicates marked heterogeneity between the T cells in these lymphoid tissues (8)

The biological role of the AP positive sub-

population of T cells is unknown. On bovine thymocytes a surface antigen (BTA) has been identified, which is expressed in less than one per cent of peripheral lymphocytes. In the thymic lymph and venous blood their numbers are much greater thus indicating that BTA positive lymphocytes in the periphery might be recent migrants (26) The AP positive T cells could also be recent migrants and thus T 1 cells. However these AP positive lymphocytes could be found in equal numbers in thymic arterial and venous blood (19) and peripheral lymphoid organs (Table 1) They were also seen in the lymph, and thus they are probably recirculating cells. They were relatively sensitive to ATS, decreased slowly after thymectomy and were located in almost equal numbers in all lymphoid tissues (Table 1) These observations are not in accordance with the properties of mouse T 1 lymphocytes (17) It would be tempting to suggest that they are suppressor cells (6) which have been shown to be a small subpopulation of T cells (4 6, 11) Thymocytes with suppressor activity have been observed to be spleen localizing after artifical peripheralization (7) This is also the property of an AP positive subpopulation of thymocytes which seems to be able to continue living outside the thymus (18) However in guinea pigs suppressor cells are sensitive to cyclophosphamide (16) which in this study induced no change in the number of AP positive lymphocytes.

The observations in this study provide further evidence that more than one cell line exists in the differentiation of thymocytes. The observations also suggest that there is heterogeneity among peripheral AP positive T cells However the biological function of AP positive subpopulation of guinea pig peripheral T cells remains to be clarified.

W wish to thank Mrs *Marja I gman* and Mrs *I ma Mäenpää* for excellent technical assistance.

REFERENCES

1 *Andersson L. C Nordling, S & Häyry P* Electrophoretic fractionation of guinea pig lymphocytes Evidence for different subsets of T and B cells in spleen and lymph node. J Immunol. *114* 1226–1229 1975

2. *Dineen J K. & Adams D B.* The effect of long-term lymphatic drainage on the lymphomyeloid system in the guinea-pig. Immunology *19* 11–30 1970.

3. *Droege W & Zucker R.* Lymphocyte subpopulations in the thymus. Transplant. Rev *25* 3–25 1975

4 *Feldmann M., Beverley P C. L., Dunkley M & Kontiainen S.* Different Ly antigen phenotypes of in vitro induced helper and suppressor cells. Nature 258 614–615, 1975

5 *Fishman, W H* Perspectives on alkaline phosphatase isoenzymes. Amer J Med. *56* 617–650, 1974

6. *Gershon R. K.* T cell control of antibody production. Contemp. Top. Immunobiol. *3* 1–40, 1974.

7 *Gershon R. K. Liebhaber E. M & Kondo K.* Immuno-regulatory role of spleen localizing thymocytes. J Immunol. *112* 546–554 1974

8. *Goldschneider I.* Antigenic relationship between bone marrow lymphocytes cortical thymocytes and a subpopulation of peripheral T cells in the rat Description of a bone marrow lymphocyte antigen. Cell. Immunol. *24* 289–307 1976.

9 *Huber B., Cantor H Shen F W & Boyse E A.* Independent differentiative pathways of Ly1 and Ly23 subclasses of T cells. Experimental production of mice deprived of selected T cell subclasses. J exp. Med. *144* 1128–1133 1976

10. *Johansen K. S Johansen T S & Talmage D W* T cell rosette formation in primates, pigs, and guinea-pigs. J Allergy Clin. Immunol *54* 86–93 1974

11 *Katz S I Parker D Sommer G & Turk J L.* Suppressor cells in normal immunisation as a basic homeostatic phenomenon Nature *248* 612–614 1974

12. *Kouvalainen, K.* Species and organ dependence of alkaline phosphatase activity in lymphatic tissues. A histochemical, biochemical and electrophoretic study Histochem J *3* 55–69 1971

13 *Merikanto J., Ruuskanen O & Thomasson, B.* Intrasternal thymectomy in guinea-pig. Transplantation *19* 72 74, 1975.

14 *Pearse A G E* Histochemistry 3 ed. vol. 1 J and A Churchill Ltd., London, 1968 p. 710.

15 *Polak L. & Turk, J L.:* Studies on the effect of systemic administration of sensitizers in guinea-pigs with contact sensitivity to inorganic metal compounds. III. The effect of immunosuppressive agents on enhancing the unresponsiveness of already sensitized animals

and an investigation of the action of the epicutaneous test within twenty four hours. Clin. Exp Immunol 4 4?3–434 1969

16 *Polak L. & Turk J L.* Reversal of immunological tolerance by cyclophosphamide through inhibition of suppressor cell activity Nature 249 654–656 1974

17 *Raff M C & Cantor H* Subpopulations of thymus cells and thymus-derived lymphocytes. In *Amos B* (Ed.) Progress in Immunology Academic Press New York, 1971 p. 83–93

18 *Ruuskanen O* Subpopulations of guinea pig thymocytes. Different distribution patterns of alkaline phosphatase positive and negative autologous thymocytes. Cell. Immunol *15* 246–254 1975

19 *Ruuskanen O & Kouvalainen K* The fate of thymocytes. A study of kinetics of thymocytes using high alkaline phosphatase activity as an endogenous label Scand. J Haemat. 9 174–185 1972

20 *Ruuskanen O & Kouvalainen K* Differentiation of thymus and thymocytes. A study in foetal guinea-pigs using alkaline phosphatase as a label of thymocytes. Immunology *26* 187–195 1974

21 *Shortman K von Boehmer H., Lipp J & Hopper K* Subpopulations of T lymphocytes. Physical separation, functional specialization and differentiation pathways of sub-sets of thymocytes and thymusdependent peripheral lymphocytes. Transplant. Rev *25* 163–210, 1975

2? *Soppi E Ruuskanen O Eskola J., Frilh, J & Kouvalainen K* Two subpopulations of guinea pig thymocytes with different maturation stages. Immunology *33* 343–350, 1977

23 *Stadecker M J Bishop G & Wortis H H* Rosette formation by guinea-pig thymocytes and thymus-derived lymphocytes with rabbit red blood cells. J Immunol. *111* 1834–1837 1973

24 *Thomasson B Ruuskanen O & Merikanto J* Spinal anaesthesia in the guinea pig Lab. Anim. *8* ?41–244 1974

25 *Turk J L. & Poulter L. W.* Selective depletion of lymphoid tissue by cyclophosphamide. Clin. Exp Immunol. *10* 285–296 1972

26 *Williams R M Chanana A D., Cronkite E. P & Waksman B H.* Antigenic markers on cells leaving calf thymus by way of the efferent lymph and venous blood. J Immunol. *106* 1143–1146 1971

27 *Wilson A B & Coombs R. R A* Rosette-formation between guinea pig lymphoid cells and rabbit erythrocytes—a possible T cell marker Int. Arch. Allergy *44* 544–552, 1973

Acta path. microbiol. scand Sect. C, 85 373–380 1977

INHIBITORY EFFECT OF HUMAN MONONUCLEAR PHAGOCYTES ON DNA SYNTHESIS IN STIMULATED LYMPHOCYTES

G Ungaard and J Lamvik

University of Trondheim, Department of Medicine, Section for haematology and immunology Trondheim, Norway

Ungaard, G & Lamvik, J Inhibitory effect of human mononuclear phagocytes on DNA synthesis in stimulated lymphocytes. Acta path. microbiol. scand. Sect C, 85 373–380 1977

Human mononuclear phagocytes cultured in vitro revealed an inhibitory influence on DNA synthesis, as measured by ³H thymidine incorporation, in lymphocytes stimulated by a lectin (PHA) soluble antigen (PPD) and allogenic lymphocytes The inhibitory effect increased with increasing ratio of macrophages to lymphocytes, and was positively related to the differentiation of monocytes to macrophages. The inhibitory ability appeared to have no connection with the histocompatibility gene complex The culture medium of macrophages cultured with and without lymphocytes showed no inhibitory effect

Key words Lymphocytes macrophages DNA synthesis PHA PPD MLC.

G. Ungaard, Trondheim Regional Hospital, Department of Medicine Section for Haematology and Immunology N-7000 Trondheim, Norway

Received 24.iii.77 Accepted 31 77

The mononuclear phagocyte system comprises cells which are circulating (monocytes) or tissue fixed (macrophages) and which have their origin in the bone marrow They have a wide variety of functions (a) Their ability to eliminate microorganisms by phagocytosis, killing and digestion is probably essential for protection against infections. (b) The lymphocyte response to antigen is influenced by macrophages via several mechanisms release of soluble factors which promote viability of the lymphocytes (6) processing and presenting of antigen to lymphocytes (14 15 16) release of soluble factors that regulate cell proliferation (3) Both stimulatory (4) and inhibitory (5 10) biological activities have been demonstrated. Even the surface of heat-killed macrophages has been reported to have an inhibitory effect on antigen-induced proliferation in lymphocytes (13) (c) There is convincing evidence for a cytotoxic effect of macrophages on malignant cells. The cytotoxicity may be a lytic effect, causing destruction of the target cells, usually assayed by measuring release of radioactive chromium from prelabelled cells (7 8) In addition, it may be a growth-inhibitory effect, usually evaluated by inhibition of DNA synthesis (1 9)

Most of the research concerning macrophages has been performed in non-human systems. This article presents results leading up to an in vitro model for testing the inhibitory effects of human mononuclear phagocytes on DNA synthesis in proliferating cells.

MATERIALS AND METHODS

Separation techniques Venous blood from healthy adults was defibrinated in Erlenmeyer flasks, using a glass stirrer (19) The mononuclear cells were isolated by centrifugation on Isopaque/Ficoll, as described by *Bøyum* (2) The defibrinated blood was diluted half and half with isotonic saline, layered on Isopaque/Ficoll and centrifuged at 400 G for 35 min. The cell layer on Isopaque/Ficoll, containing mostly mononuclear cells, was removed by pipette and the cells were washed twice in Hanks balanced salt solution (BBS)

Macrophage cultures The cells were suspended in culture medium consisting of 75 per cent RPMI 1640 (Gibco Bio-Cult, Glasgow Scotland) and 25 per cent pooled human A serum supplemented by 0 1 mg l-glutamin per ml Gentamycin was added to the RPMI 1640 in a concentration of 40 mg/l Cell suspensions containing 4×10^6 cells per ml were applied either directly into plastic petri dishes (Nunclon) with a diameter of 50 mm total area at the bottom 19 6 cm^2 or to heat sterilized glass coverslips placed in duplicate in petri dishes. Rectangular coverslips of two different sizes were used viz. 11×35 mm or 10.5×22 mm. One half ml was applied to the larger cover slips 0 3 ml to the smaller and 2.5 ml to the petri dishes where macrophages were grown without coverslips. The dishes were incubated in a National CO_2 incubator at 37 C with 7.5 per cent CO_2 in air and with 100 per cent humidity The cells were allowed to attach to the plastic or glass surface during an incubation period of 90 min. The medium containing non adherent cells was then removed and 2.5 ml of fresh culture medium was added to each dish. After one day and after four days the culture medium was changed, unless the cells had not already been used in experiments.

Lymphocyte cultures For the study of DNA synthesis in lymphocytes, mononuclear blood cells were suspended in culture medium consisting of 80 per cent RPMI 1640 and 20 per cent pooled human AB serum The mixture of l-glutamin and gentamycin was added as already described The suspension were applied to petri dishes (Ø 50 mm) with mononuclear phagocytes covering either the bottom of the dishes or covering coverslips placed in the dishes. Two and a half ml of the cell suspension with a cell concentration of 10^6 cells per ml was added to each dish after removal of the macrophage culture medium. The control dishes contained no precultured mononuclear phagocytes. In some of the experiments the lymphocytes were cultured for three days in Kimax tubes before application to petri dishes.

Stimulation of lymphocytes. In most of the experiments lyophilized phytohaemagglutinin (PHA) from Gibco reconstituted with 10 ml distilled water was used in a quantity of 20 µl per ml culture medium. For antigen stimulation, purified tuberculin (PPD) was added to a final concentration of 2 µg per ml Lymphocytes for the latter experiments were separated from persons who showed a positive tuberculin reaction (Pirquet > 10 mm) Lymphocytes were stimulated by allogeneic lymphocytes in some of the experiments. Mixed lymphocyte cultures (MLC) were prepared by mononuclear cells from two individuals, making a final concentration of 5×10^5 cells per ml of each genotype.

Assay of DNA synthesis The cultures stimulated with PHA were incubated for four days before harvesting if not otherwise stated. The PPD-stimulated cultures and the MLC were in most experiments harvested on the sixth day Eighteen hours before harvesting 0.5 µCi ^3H thymidine was added per ml culture medium. The cells were harvested using a Titertek multiple cell harvester (Flow Scotland) and the radioactivity incorporated into the cells was measured in a liquid scintillation counter (Isocap/300 Nuclear Chicago USA) The data are given in net counts.

In order to ensure that all the ^3H thymidine incorporated in the cells were counted, the macrophages were removed from the plastic or glass surface by a combination of osmotic lysis by distilled water and mechanical forces, and harvested with the multiple cell harvester

EXPERIMENTS AND RESULTS

Inhibition of ^3H thymidine incorporation in PHA stimulated lymphocytes When PHA stimulated lymphocytes were added to allogeneic macrophages, precultured in vitro for eight days, the ^3H thymidine incorporation was found constantly to be inhibited, the inhibition being related to the area of the petri dish covered by macrophages (Fig 1) The area was varied by culturing macrophages on coverslips of different sizes and in different numbers placed in the petri dishes (one small – 2 8 cm^2 one large – 3.9 cm two large – 7 7 cm three large – 11 6 cm) In some of the dishes the macrophages grew at the bottom (19 6 cm) without coverslips. The density of the adherent cells was approximately constant, thus making the area covered by macrophages a proportional expression of the number of macrophages.

In order to investigate whether inhibition could be due to absorption of PHA by macrophages, various doses of PHA were tested

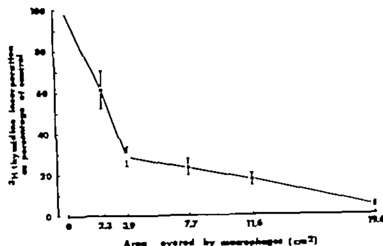


Fig 1 Inhibition of ³H thymidine incorporation in PHA-stimulated lymphocytes by macrophages precultured in vitro for eight days. The graph show the mean and SE of six experiments carried out in duplicate.

(Fig. 2) A fifty-fold difference in the PHA dose did not alter the inhibition, thus making PHA absorption an unlikely explanation. To confirm this, the following experiment was designed. Two and a half ml of complete medium with 20 μl reconstituted PHA per ml was added to petri dishes, each with three large macrophage-covered coverslips. After a 15 min incubation period, the medium was removed and centrifuged at 18,000 *G* for 10 min. Lymphocytes were suspended in the supernatant. Controls were lymphocytes in fresh medium supplied with PHA. The ³H thymidine incorporation in lymphocytes cultured in macrophage-absorbed medium did not differ from ³H thymidine incorporation in controls. The mean value and SE of six experiments as percentage of controls was 100 ± 4.

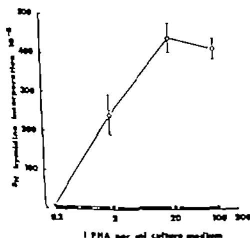


Fig 2 Dose-response curve of ³H thymidine incorporation in PHA-stimulated lymphocytes (● — ●) with macrophages covering the bottom (19.6 cm²) (○ — ○) without macrophages. The macrophages were precultured for eight days. The values are means ± SE of four experiments carried out in duplicate.

The duration of culturing PHA-stimulated lymphocytes in the presence of macrophages did not influence the inhibition (Fig 3). A lymphocyte suspension supplied with PHA was added to petri dishes with a macrophage monolayer covering the bottom (19.6 cm²). Duplicate cultures were harvested at different times. For all the intervals tested (2d–7d) ³H thymidine incorporation was inhibited to less than 2 per cent of the controls.

Macrophages precultured in vitro for four days showed the same inhibitory effect on ³H thymidine incorporation in PHA-stimulated lymphocytes as macrophages precultured for eight days (Table 1 and Fig. 1).

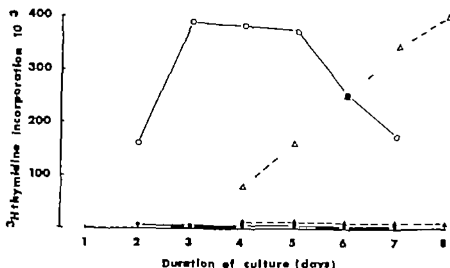


Fig 3 Kinetics of ³H thymidine incorporation in lymphocytes stimulated with 20 µl PHA per ml culture medium (——) or with 2 µg PPD per ml culture medium (– – –) The lymphocytes were cultured with macrophages covering the bottom of the dish (●——● ▲– – –▲) or without macrophages (○——○ △– – –△) The macrophages were precultured for eight days. The values represent one experiment carried out in triplicate.

TABLE 1 *Inhibition of ³H Thymidine Incorporation in PHA-Stimulated Lymphocytes by* Allogeneic *and* Autologous *Macrophages Precultured in Vitro for four Days*

Area covered by macrophages	³H thymidine incorporation as percentage of controls	
	Allogeneic cells	Autologous cells
0 (controls)	100	100 b
2 3 cm²	77 ± 7	84 ± 5
3 9 cm²	31 ± 3	46 ± 9
7 7 cm²	22 ± 2	23 ± ?
11 6 cm²	18 ± 2	2? ± 3
19 6 cm²	11 ± 3	13 ± 4

Values are means ± SE of six experiments carried out in duplicate
ᵃH thymidine incorporation in controls
306 (SE 25) × 10³ c p m.
b 303 (SE 4?) × 10³ c p m.

Inhibition of ³H thymidine incorporation in PPD-stimulated lymphocytes and in mixed lymphocyte cultures Macrophages were also found to inhibit the ³H thymidine incorporation in lymphocytes stimulated by PPD or by allogeneic lymphocytes (Table 2)

In order to increase the ³H thymidine incorporation induced by allogeneic lymphocytes, the mixed lymphocyte cultures were incubated for three days in Kimax tubes before transfer to petri dishes.

The dose of PPD used was 2µg per ml culture medium The dose was not critical for the inhibition of ³H thymidine incorporation observed (Fig 4) Nor did the inhibitory phenomenon depend critically on the time of culturing, as long as the registration was done within the interval which gave a good response in the controls (Fig 3)

Influence of in vitro preculturing of mononuclear phagocytes on their ability to inhibit ³H thymidine incorporation in PHA stimulated lymphocytes In order to get an idea of how long macrophages had to be in the presence of lymphocytes to inhibit their ³H thymidine incorporation the following experiment was designed Macrophages were precultured in petri dishes. After four days, freshly separated allogeneic lymphocytes were added to some of the dishes and stimulated with PHA. PHA stimulated lymphocytes from the same separation were cultured for three days in Kimax tubes. Then the contents of the culture tubes were applied to the rest of the macrophage cultures. One hour later ³H thymidine was added to all the cultures, and

TABLE 2. *Inhibition of ³H Thymidine Incorporation in PPD-stimulated Lymphocytes and in Mixed Lymphocyte Cultures by Allogeneic Macrophages Precultured in Vitro for five Days*

Area covered by macrophages	³H thymidine incorporation as percentage of controls	
	PPD-stimul.	MLC
0 (controls)	100 ᵃ	100 ᵇ
2.3 cm²	81 ± 4	90 ± 6
3.9 cm²	52 ± 5	78 ± 7
7.7 cm²	27 ± 4	57 ± 7
11.6 cm²	17 ± 4	40 ± 6
19.6 cm²	3 ± 1	5 ± 1

Mixed lymphocyte cultures were incubated for three days in Kimax tubes before transfer to macrophages. PPD-stimulated lymphocytes were in the petri dishes during the whole stimulation period. The values are means ± SE of six experiments carried out in duplicate.
ᵃ ³H thymidine incorporation in controls
185 (SE 30) × 10³ c.p.m.
ᵇ 208 (SE 25) × 10³ c.p.m.

after a period of 18 hours they were all harvested. Macrophages had a quite immediate effect on ³H thymidine incorporation (Table 3). There was no difference between the inhibition when macrophages were present during the whole PHA stimulation period compared with the inhibitory effect when stimulated lymphocytes were exposed to macrophages during the ³H thymidine incubation period. The latter procedure was used in the next experiments designed to show the relationship between time of in vitro preculturing of mononuclear phagocytes and their inhibitory ability.

PHA-stimulated lymphocytes were cultured for three days in Kimax tubes before the cultures were applied to petri dishes containing allogeneic or autologous mononuclear phagocytes which had been precultured in vitro for 90 min, three days or seven days. The cultured cells were washed twice in complete medium to remove non-adherent cells before addition of lymphocytes. One hour later ³H thymidine was added, and after a period of 18 hours the cultures were harvested. The

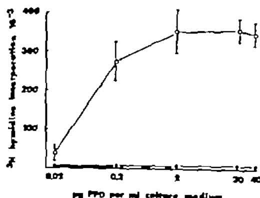


Fig. 4. Dose-response curve of ³H thymidine incorporation in PPD-stimulated lymphocytes (●—●) with macrophages covering the bottom (○—○) without macrophages. The macrophages were precultured for eight days. The values are means ± SE of four experiments carried out in duplicate.

results show that the inhibitory ability of mononuclear phagocytes increased with the time of in vitro preculturing (Table 4).

Inhibitory phenomenon and the histocompatibility gene complex. There was no differ

TABLE 3. *Inhibition of ³H Thymidine Incorporation in PHA-Stimulated Lymphocytes by Macrophages being Present to Lymphocytes at Different Intervals*

Lymphocytes cultures in the presence of macrophages	³H thymidine incorporation as percentage of controls
During the whole stimulation period	10 ± 3
Controls (without macrophages)	100 ᵃ
During the ³H thymidine incubation period	9 ± 2
Controls (without macrophages)	100 ᵇ

The whole area (19.6 cm²) at the bottom of the petri dishes was covered by macrophages. At the time of harvesting macrophages in both variants had been cultured in vitro for eight days. The values are means ± SE of six experiments carried out in duplicate.
ᵃ ³H thymidine incorporation in controls
254 (SE 38) × 10³ c.p.m.
ᵇ 202 (SE 48) × 10³ c.p.m.

TABLE 4 *Inhibition of ³H Thymidine Incorporation in PHA-Stimulated Lymphocytes by* Autologous *and* Allogeneic *Mononuclear Phagocytes Precultured in Vitro for Various Times*

Time of macrophage preculturing	³H thymidine incorporation as percentage of controls	
	Allogeneic cells	Autologous cells
Controls	100	100
90 min	83 ± 8	84 ± 6
3 days	38 ± 4	37 ± 3
7 days	9 ± 2	—

Mononuclear phagocytes covered the total bottom area (19 6 cm²) of the petri dishes. The PHA stimulated lymphocytes were present to monocytes/macrophages only during the ³H thymidine incor poration period The values are means ± SE of six experiments carried out in duplicate

³H thymidine incorporation in controls 185 (SE 30) × 10³ c.p.m.

TABLE 5 *³H Thymidine Incorporation in PHA-Stimulated Lymphocytes Suspended in Supernatants of Culture Media*

Cultured media tested	³H thymidine incorporation as percentage of controls
a. Supernatants of macrophage medium macrophages had been cultured in the medium from the 8th to the 12th day	100 ± 5
b Supernatants of lymphocyte medium PHA-stimulated lymphocytes had been cultured in the medium for four days	99 ± 8
c. Supernatants of medium from macrophages and PHA stim ulated lymphocytes cultured together (a + b)	102 ± 9
d. Controls (fresh medium)	100

The values are means ± SE of four experiments carried out in quadruplicate.

³H thymidine incorporation in controls 140 (SE 8) c.p.m. × 10³

ence between the inhibitory ability of autologous and allogeneic mononuclear phagocytes, thus indicating that the histocompat ibility gene complex is not involved in the phenomenon (Tables 1 and 4)

Influence of supernatants from mononuclear leucocyte cultures on ³H thymidine incorporation in PHA stimulated lymphocytes The supernatants to be tested were high speed centrifuged media from the following cultures macrophages, PHA stimulat ed lymphocytes, and macrophages and PHA stimulated lymphocytes cultured together The media were incubated with the various cells for four days. The supernatants of the media were tested with regard to a possible inhibitory influence on ³H thymidine incor poration in PHA stimulated lymphocytes (test lymphocytes) At the start of the ex periment, the test lymphocytes, which had been precultured in Kimax tubes supplied with PHA for three days were centrifuged and resuspended in the various supernatants or in fresh medium as control. ³H thymidine was added immediately and the cells were harvested 18 hours later

The various supernatants showed no inhibitory effect on ³H thymidine incorporation in the PHA-stimulated lymphocytes.

DISCUSSION

³H thymidine incorporation in lymphocytes induced by different stimuli was found constantly to be inhibited by mononuclear blood cells adherent to glass or plastic surfaces. We assume that the effector cells are the mononuclear phagocytes, because adherent lym phocytes and granulocytes would have detached during the long preculturing period of up to eight days, leaving a pure culture of mononuclear phagocytes at the start of the experiment

The inhibitory effect was related to the size of the area covered by macrophages. The density of adherent cells was approximately constant in each experiment making the ma crophage covered area a proportional expression of the number of macrophages. Accord

ingly the results show increased inhibition with increasing numbers of macrophages. This is in agreement with the findings of *Waldman* & *Gottlieb* (18). However in their system of rat cells peritoneal exudate cells were shown to have a dual influence on "spontaneous" ³H thymidine incorporation in spleen lymphoid cells depending on the ratio of macrophages to lymphocytes. A small ratio caused stimulation, while a higher ratio caused inhibition. In this connection it is important to remember that in our experiments no attempts were made to purify the lymphocyte cultures. The separated mononuclear cells were used without further isolation. They contained up to 25 per cent monocytes. In a few experiments where only a small coverslip with macrophages was placed in the dishes, a slight stimulatory effect was found pointing towards a dual effect related to the quantity of macrophages. This needs further investigation.

The inhibition was found to be a quite immediate effect in our experiments. There was no difference in inhibition when macrophages were present to lymphocytes from the beginning of the lymphocyte culture as compared to when macrophages were supplied three days later at the time of ³H thymidine addition. However these results are not conclusive, because the high ratio of macrophages to lymphocytes might mask a possible effect on the early events in lymphocyte proliferation.

The inhibitory effect was positively related to the preculturing time of the mononuclear phagocytes. In vitro culturing of human monocytes for eight days has been shown to give macrophage properties to the cells with regard to structure and function (17 19). It is interesting to note that this development was paralleled by the development of inhibitory ability on ³H thymidine incorporation in lymphocytes.

The inhibitory effect does not appear to be connected with the histocompatibility gene complex, because there was no difference in autologous and allogeneic systems.

Different mechanisms might explain the inhibitory effect of macrophages on immunogenic and mitogenic induction of ³H thymidine incorporation in lymphocytes. Elimination of the stimulus by macrophage absorption is very unlikely as regards PHA this was excluded by experiments. Phagocytosis of the lymphocytes by macrophages might explain the results, but investigations with inverted phase contrast microscopy of living cells in petri dishes, and with fluorescent microscopy of living cells in microchambers after acridin orange staining resulted in no phagocytosis being detected.

The most likely explanation of the inhibited H thymidine incorporation is a cytotoxic effect causing either rapid lysis of the lymphocytes or just growth inhibition. The cytotoxicity may be executed either by membrane contact or by a humoral factor. In murine systems culture medium from peritoneal exudate cell cultures has been shown to inhibit H thymidine incorporation in lymphocytes (10 18). The effector was found to be a stable, dialysable factor secreted by macrophages. However other investigators (11 12) have demonstrated in similar systems that the factor was cold thymidine, a degradation product from DNA of cells phagocytosed and digested by macrophages.

We found no inhibitory effect of culture supernatants. These experiments exclude malnutrition and cold thymidine as the cause of inhibition. It is therefore justifiable to conclude that inhibited ³H thymidine incorporation is a true expression of inhibited DNA synthesis in this system.

The experiments with culture supernatants might lead to the conclusion that contact is essential for inhibition. However from the graph of the growth inhibition plotted against the macrophage-covered area (Fig 1) it will be seen that there is no linear relationship. When macrophages cover one fifth of the area leaving the lymphocytes to sediment on free plastic on *four-fifths* of the area, DNA synthesis is still reduced to 29 per cent of the control. This brings a humoral factor into consideration, and in view of the findings with supernatants from macrophage cul-

TABLE 4 *Inhibition of ^{3}H Thymidine Incorporation in PHA-Stimulated Lymphocytes by* Autologous *and* Allogeneic *Mononuclear Phagocytes Precultured in Vitro for Various Times*

Time of macrophage preculturing	^{3}H thymidine incorporation as percentage of controls	
	Allogeneic cells	Autologous cells
Controls	100 [a]	100 [a]
90 min	83 ± 8	84 ± 6
3 days	38 ± 4	37 ± 3
7 days	9 ± 2	—

Mononuclear phagocytes covered the total bottom area (19 6 cm²) of the petri dishes. The PHA stimulated lymphocytes were present to monocytes/macrophages only during the ^{3}H thymidine incorporation period The values are means ± SE of six experiments carried out in duplicate

[a] ^{3}H thymidine incorporation in controls = 185 (SE 30) × 10³ c.p.m

TABLE 5 *^{3}H Thymidine Incorporation in PHA-Stimulated Lymphocytes Suspended in Supernatants of Culture Media*

Cultured media tested	^{3}H thymidine incorporation as percentage of controls
a. Supernatants of macrophage medium macrophages had been cultured in the medium from the 8th to the 12th day	100 ± 5
b Supernatants of lymphocyte medium PHA-stimulated lymphocytes had been cultured in the medium for four days	99 ± 8
c Supernatants of medium from macrophages and PHA-stimulated lymphocytes cultured together (a + b)	102 ± 9
d. Controls (fresh medium)	100

The values are means ± SE of four experiments carried out in quadruplicate.

H thymidine incorporation in controls 140 (SE 8) c.p m. × 10³

ence between the inhibitory ability of autologous and allogeneic mononuclear phagocytes thus indicating that the histocompatibility gene complex is not involved in the phenomenon (Tables 1 and 4)

Influence of supernatants from mononuclear leucocyte cultures on ^{3}H thymidine incorporation in PHA stimulated lymphocytes The supernatants to be tested were high speed centrifuged media from the following cultures macrophages, PHA stimulated lymphocytes, and macrophages and PHA stimulated lymphocytes cultured together The media were incubated with the various cells for four days. The supernatants of the media were tested with regard to a possible inhibitory influence on ^{3}H thymidine incorporation in PHA stimulated lymphocytes (test lymphocytes) At the start of the experiment, the test lymphocytes, which had been precultured in Kimax tubes supplied with PHA for three days, were centrifuged and resuspended in the various supernatants or in fresh medium as control. ^{3}H thymidine was added immediately and the cells were harvested 18 hours later

The various supernatants showed no inhibitory effect on ^{3}H thymidine incorporation in the PHA stimulated lymphocytes.

DISCUSSION

^{3}H thymidine incorporation in lymphocytes induced by different stimuli was found constantly to be inhibited by mononuclear blood cells adherent to glass or plastic surfaces. We assume that the effector cells are the mononuclear phagocytes, because adherent lymphocytes and granulocytes would have detached during the long preculturing period of up to eight days, leaving a pure culture of mononuclear phagocytes at the start of the experiment

The inhibitory effect was related to the size of the area covered by macrophages. The density of adherent cells was approximately constant in each experiment, making the macrophage covered area a proportional expression of the number of macrophages. Accord

ingly the results show increased inhibition with increasing numbers of macrophages. This is in agreement with the findings of *Waldman* & *Gottlieb* (18). However in their system of rat cells peritoneal exudate cells were shown to have a dual influence on spontaneous ³H thymidine incorporation in spleen lymphoid cells depending on the ratio of macrophages to lymphocytes. A small ratio caused stimulation, while a higher ratio caused inhibition. In this connection it is important to remember that in our experiments no attempts were made to purify the lymphocyte cultures. The separated mononuclear cells were used without further isolation. They contained up to 25 per cent monocytes. In a few experiments where only a small coverslip with macrophages was placed in the dishes, a slight stimulatory effect was found, pointing towards a dual effect related to the quantity of macrophages. This needs further investigation.

The inhibition was found to be a quite immediate effect in our experiments. There was no difference in inhibition when macrophages were present to lymphocytes from the beginning of the lymphocyte culture as compared to when macrophages were supplied three days later at the time of ³H thymidine addition. However these results are not conclusive, because the high ratio of macrophages to lymphocytes might mask a possible effect on the early events in lymphocyte proliferation.

The inhibitory effect was positively related to the preculturing time of the mononuclear phagocytes. In vitro culturing of human monocytes for eight days has been shown to give macrophage properties to the cells with regard to structure and function (17, 19). It is interesting to note that this development was parallelled by the development of inhibitory ability on ³H thymidine incorporation in lymphocytes.

The inhibitory effect does not appear to be connected with the histocompatibility gene complex, because there was no difference in autologous and allogeneic systems.

Different mechanisms might explain the inhibitory effect of macrophages on immunogenic and mitogenic induction of ³H thymidine incorporation in lymphocytes. Elimination of the stimulus by macrophage absorption is very unlikely; as regards PHA this was excluded by experiments. Phagocytosis of the lymphocytes by macrophages might explain the results, but investigations with inverted phase contrast microscopy of living cells in petri dishes, and with fluorescent microscopy of living cells in microchambers after acridin orange staining, resulted in no phagocytosis being detected.

The most likely explanation of the inhibited ³H thymidine incorporation is a cytotoxic effect causing either rapid lysis of the lymphocytes or just growth inhibition. The cytotoxicity may be executed either by membrane contact or by a humoral factor. In murine systems, culture medium from peritoneal exudate cell cultures has been shown to inhibit ³H thymidine incorporation in lymphocytes (10, 18). The effector was found to be a stable, dialyzable factor secreted by macrophages. However other investigators (11, 12) have demonstrated in similar systems that the factor was cold thymidine, a degradation product from DNA of cells phagocytosed and digested by macrophages.

We found no inhibitory effect of culture supernatants. These experiments exclude malnutrition and cold thymidine as the cause of inhibition. It is therefore justifiable to conclude that inhibited ³H thymidine incorporation is a true expression of inhibited DNA synthesis in this system.

The experiments with culture supernatants might lead to the conclusion that contact is essential for inhibition. However from the graph of the growth inhibition plotted against the macrophage-covered area (Fig 1) it will be seen that there is no linear relationship. When macrophages cover one-fifth of the area leaving the lymphocytes to sediment on free plastic on four fifths of the area, DNA synthesis is still reduced to 29 per cent of the control. This brings a humoral factor into consideration, and in view of the findings with supernatants from macrophage cul-

TABLE 4 *Inhibition of ³H Thymidine Incorporation in PHA-Stimulated Lymphocytes by* Autologous *and* Allogeneic *Mononuclear Phagocytes Precultured in Vitro for Various Times*

Time of macrophage preculturing	³H thymidine incorporation as percentage of controls	
	Allogeneic cells	Autologous cells
Controls	100	100
90 min	83 ± 8	84 ± 6
3 days	38 ± 4	37 ± 3
7 days	9 ± 2	—

Mononuclear phagocytes covered the total bottom area (19 6 cm²) of the petri dishes. The PHA stimulated lymphocytes were present to monocytes/macrophages only during the ³H thymidine incorporation period. The values are means ± SE of six experiments carried out in duplicate.

³H thymidine incorporation in controls
= 185 (SE 30) × 10³ c.p.m

TABLE 5 *³H Thymidine Incorporation in PHA-Stimulated Lymphocytes Suspended in Supernatants of Culture Media*

Cultured media tested	³H thymidine incorporation as percentage of controls
a. Supernatants of macrophage medium macrophages had been cultured in the medium from the 8th to the 12th day	100 ± 5
b Supernatants of lymphocyte medium PHA-stimulated lymphocytes had been cultured in the medium for four days	99 ± 8
c. Supernatants of medium from macrophages and PHA-stimulated lymphocytes cultured together (a + b)	102 ± 9
d. Controls (fresh medium)	100

The values are means ± SE of four experiments carried out in quadruplicate.

³H thymidine incorporation in controls
140 (SE 8) c.p.m. × 10³

ence between the inhibitory ability of autologous and allogeneic mononuclear phagocytes, thus indicating that the histocompatibility gene complex is not involved in the phenomenon (Tables 1 and 4)

Influence of supernatants from mononuclear leucocyte cultures on ³H thymidine incorporation in PHA stimulated lymphocytes The supernatants to be tested were high speed centrifuged media from the following cultures macrophages, PHA stimulated lymphocytes, and macrophages and PHA stimulated lymphocytes cultured together The media were incubated with the various cells for four days. The supernatants of the media were tested with regard to a possible inhibitory influence on ³H thymidine incorporation in PHA-stimulated lymphocytes (test lymphocytes) At the start of the experiment, the test lymphocytes, which had been precultured in Kimax tubes supplied with PHA for three days, were centrifuged and resuspended in the various supernatants or in fresh medium as control. ³H thymidine was added immediately and the cells were harvested 18 hours later

The various supernatants showed no inhibitory effect on ³H thymidine incorporation in the PHA-stimulated lymphocytes.

DISCUSSION

³H thymidine incorporation in lymphocytes induced by different stimuli was found constantly to be inhibited by mononuclear blood cells adherent to glass or plastic surfaces. We assume that the effector cells are the mononuclear phagocytes, because adherent lymphocytes and granulocytes would have detached during the long preculturing period of up to eight days, leaving a pure culture of mononuclear phagocytes at the start of the experiment.

The inhibitory effect was related to the size of the area covered by macrophages. The density of adherent cells was approximately constant in each experiment, making the macrophage covered area a proportional expression of the number of macrophages. Accord

Acta path. microbiol. scand. Sect. C, 85 381–388, 1977

IMMUNO INDUCTIVE AND IMMUNO-SUPPRESSIVE INFLUENCE OF HUMAN MONONUCLEAR PHAGOCYTES CULTURED *IN VITRO*

G Unsgaard

University of Trondheim, Department of Medicine Section for Haematology and Immunology Trondheim, Norway

Unsgaard, G Immuno-inductive and immuno-suppressive influence of human mononuclear phagocytes cultured in vitro. Acta path. microbiol. scand. Sect. C, 85 381–388, 1977

DNA-synthesis, as measured by ³H thymidine incorporation, in immune lymphocytes from human blood stimulated in vitro with PPD was shown to require monocytes. No such requirement was demonstrated for PHA-induced DNA-synthesis under the same conditions. Initial packing of cells by centrifugation was beneficial for the cultures stimulated by antigen, but not for the cultures stimulated by lectin, thus indicating the necessity for contact between monocytes and lymphocytes for antigen stimulation. Monocytes and macrophages preincubated with PPD induced DNA-synthesis in lymphocytes. Monocytes were found to be at least as capable of retaining and presenting antigen as macrophages, and autologous macrophages presented antigen more efficiently than allogeneic macrophages. DNA-synthesis in lymphocytes, induced by adding PPD or PHA to the culture medium, was inhibited by a large number of monocytes. Macrophages caused inhibition under the same conditions, even a small number. DNA-synthesis in lymphocytes induced by PPD-preincubated monocytes and macrophages increased with increasing ratio of mononuclear phagocytes to lymphocytes up to a certain value. A further increase in the ratio caused inhibition, thus indicating both an immuno-inductive and an immuno-suppressive influence of mononuclear phagocytes.

Key words: Macrophages; lymphocytes; immuno-induction; immuno-suppression; ³H thymidine; PHA; PPD

G. Unsgaard, Trondheim Regional Hospital, Department of Medicine, Section for Haematology and Immunology, N 7000 Trondheim, Norway

Received 24.iii.77   Accepted 31.v.77

Blood monocytes cultured adherent to a glass or plastic surface for up to eight days will obtain macrophage characteristics with regard to structure and phagocytic capacity (16, 17). In a previous report we demonstrated that this differentiation was followed by an increasing inhibitory influence of the macrophages on lectin-induced DNA synthesis in lymphocytes (15). DNA synthesis in mixed lymphocyte cultures and in lymphocytes stimulated by a soluble antigen (PPD) was also found to be suppressed by macrophages.

However there is convincing evidence for an inductive role of macrophages in the humoral and cellular immune response. Antibody production in response to most antigens requires macrophages as antigen-focusing

tures, it might be an unstable factor Further investigations are in progress to characterize the nature of the inhibitory phenomenon.

The technical assistance of Mrs. *A Remen* and Mrs. *B Lippe* is gratefully acknowledged. The work was supported by grants from the *Norwegian Research Council for Science and the Humanities* the *Norwegian Cancer Society* and the *Norwegian Society for Fighting Cancer*

One of the authors (GU) is a research fellow of the *Norwegian Research Council for Science and the Humanities*

REFERENCES

1 *Alexander P* The functions of the macrophages in malignant disease. In *Greger W P* (Ed) Annual Review of medicine. Selected topics in the clinical sciences. Annual Reviews Inc. Palo Alto California, USA 1976 *27* 207–224

2. *Bøyum A* Separation of leukocytes from blood and bone marrow Scand. J Clin. Lab. Invest. *21* Suppl. 97 1968

3 *Calderon J & Unanue E R* Two biological activities regulating cell proliferation found in cultures of peritoneal exudate cells. Nature *253* 359–361 1975

4 *Calderon J Kiely J M Lefko J L. & Unanue E. R* The modulation of lymphocyte functions by molecules secreted by macrophages I Description and partial biochemical analysis. J Exp Med. *142* 151–164 1975

5 *Calderon J Williams R T & Unanue E R* An inhibitor of cell proliferation released by cultures of macrophages. Proc. Nat. Acad Sci. USA *71* 4273–4277 1974

6 *Chen C & Hirsch J G* The effects of mercaptoetanol and of peritoneal macrophages on the antibody-forming capacity of non adherent mouse spleen cells *in vitro* J Exp Med *136* 604–617 1972

7 *Evans R & Alexander P* Role of macrophages in tumor immunity I Co-operation between macrophages and lymphoid cells in syngeneic tumor immunity Immunol *23* 615 –626 1972.

8 *Evans R & Alexander P* Role of macrophages in tumor immunity II Involvement of a macrophage cytophilic factor during syngeneic tumor growth inhibition. Immunol. *23* 627–636 1972.

9 *Keller R.* Cytostatic elimination of syngeneic rat tumor cells *in vitro* by nonspecifically activated macrophages. J Exp. Med. *138* 625–644 1973

10 *Nelson D S* Production by stimulated macrophages of factors depressing lymphocyte transformation. Nature *246* 306–307 1973

11 *Opitz H G., Niethammer D., Lemke H Flad H D & Huget R.* Inhibition of H³ thymidine incorporation of lymphocytes by a soluable factor from macrophages. Cell. Immunol. *16* 379–388 1975

12 *Opitz H G Niethammer D Jackson R. C Lemke H Huget R & Flad, H D* Biochemical characterization of a factor released by macrophages. Cell. Immunol. *18* 70–75 1975

13 *Ptak W & Gershon R K* Immunosuppression effected by macrophage surface. J Immunol. *115* 1346–1350 1975

14 *Rosenthal A S & Shevach E. M.* Function of macrophages in antigen recognition by guinea pig T lymphocytes. I Requirement for histocompatible macrophages and lymphocytes. J Exp Med. *138* 1194–1212 1973

15 *Shevach E M & Rosenthal, A S* Function of macrophages in antigen recognition by guinea pig T lymphocytes. II Role of the macrophage in the regulation of genetic control of the immune response. J Exp Med. *138* 1212–1229 1973

16 *Unanue E. R & Calderon J* Evaluation of the role of macrophages in immune induction. Fed. Proc. *34* 1737–1742 1975

17 *Viken K E. & Ødegaard A* Phagocytosis of heat killed radiolabelled *Candida albicans* by human blood monocytes cultured *in vitro*. Acta path. microbiol scand. Sect. B, *82* 235 –244 1974

18 *Waldman S R & Gottlieb A* Macrophage regulation of DNA synthesis in lymphoid cells Effects of a soluble factor from macrophages Cell Immunol *9* 142–156 1973

19 *Ødegaard A Viken K E & Lamvik J* Structural and functional properties of blood monocytes cultures *in vitro* Acta path. microbiol scand. Sect. B *82* 223–234 1974

Assay of DNA-synthesis The cultures stimulated with PPD and the MLC were incubated for six days. The PHA-stimulated cultures were harvested on the fourth day.

Eighteen hours before harvesting 0.5 μCi H thymidine (sp act. 26 Ci/m mol) was added per ml. The cells were harvested in the following way: The medium with non-adherent cells was transferred to Kimax tubes with pipette. Two and a half ml of distilled water was then added to each dish, and adherent cells were removed from the surface by a combination of osmotic lysis and jet streams from the pipette. The suspension was transferred to Kimax tubes. Another 2.5 ml of distilled water was added and the procedure was repeated. Finally the nuclear material was trapped in filters (Skatron A/S, Lierbyen, Norway) by a Titertec multiple cell harvester (Flow Scotland). The filters were applied to vials, and the radioactivity incorporated was counted in a liquid scintillation counter (Locap 1300, Nuclear Chicago, USA). The data represent net counts.

It was shown that the hypotonic milieu during harvesting did not influence the radioactivity as measured by counts per minute. Lymphocytes (Ly) stimulated by PHA were cultured in Kimax tubes and supplied with ³H thymidine 18 hours before harvesting. Some tubes were harvested at the set time, to others 7.5 ml distilled water was added, and they were harvested two or four hours later. The experiments carried out in triplicate showed no reduction in the nuclear material radioactivity after two hours in extreme hypotonicity. After four hours a 20 per cent reduction was found. The harvesting procedure never exceeded two hours.

EXPERIMENTS AND RESULTS

Influence of the culture conditions on H thymidine incorporation in lymphocytes stimulated by PHA, PPD and allogeneic lymphocytes. Lymphocytes (Ly) from the same separation were cultured either in Kimax tubes, in petri dishes, or for part of the period in Kimax tubes and for the rest of the period in petri dishes. The cell suspensions were transferred gently from tube to dish by pipette. Following PHA stimulation ³H thymidine incorporation was greatest in the petri dish cultures and smallest in the Kimax cultures, while the values for cell samples cultured partly in Kimax tubes and partly in petri dishes were in between (Table 1). This was found in each of six experiments. When stimulated by PPD or allogeneic lymphocytes, the ³H thymidine incorporation was smallest in Kimax tubes, significantly greater in petri dish cultures, but greatest in cell samples cultured in Kimax tubes for half of the period and in petri dishes for the rest of the period (Table 2).

TABLE 1. *Influence of Different Culture Conditions on DNA Synthesis in Lymphocytes Stimulated by PHA*

Culture conditions	³H thymidine incorporation c.p.m. × 10⁻³
Kimax, 4 d	134 ± 30
Kimax, 3 d/Petri dish, 1 d	195 ± 41
Kimax, 2 d/Petri dish, 2 d	265 ± 44
Petri dish, 4 d	293 ± 51

The values are means ± SE of six experiments carried out in triplicate.

TABLE 2. *Influence of Different Culture Conditions on DNA Synthesis in Lymphocytes Stimulated by PPD and Allogeneic Lymphocytes*

Culture conditions	³H thymidine incorporation c.p.m. × 10⁻³	
	PPD*	MLC**
Kimax, 6 d	93 ± 17	39 ± 7
Kimax, 3 d/Petri dish, 3 d	254 ± 32	214 ± 18
Petri dish, 6 d	175 ± 23	72 ± 17

The values are means ± SE of six or eight experiments carried out in triplicate.
* n = 8
** n = 6

H thymidine incorporation in PHA and PPD-stimulated lymphocytes with varying degrees of monocyte contamination. The contamination of adherent cells in the lymphocyte cultures was reduced from an average of 19 per cent to an average of 4 per cent by permitting the cells to attach to a plastic surface during an incubation period of 90 min. After a repetition of this procedure the contamination averaged 0.5 per cent. Suspensions of lymphocytes were adjusted to 10⁶ cells per ml and applied to petri dishes. The cells were stimulated by PHA or PPD. The PHA in-

cells for T B lymphocyte interaction (1 14) Proliferation in immune T cells requires presentation of the specific antigen by macrophages (11) It has been suggested that this antigen presentation is dependent on membrane contact between lymphocytes and macrophages, and a requirement for histocompatibility has been demonstrated in a guinea pig system (9 12 13) Murine macrophages have been found to secrete non-dialysable molecules that increase the proliferation in lectin stimulated lymphocytes, and even molecules that induce proliferation in spleen cells and thymocytes without addition of lectin or antigen (2, 3 4)

The aim of this work was to investigate the relationship between the immuno-inductive and immuno-suppressive role of mononuclear phagocytes in a human *in vitro system*. One question to be answered was whether the ratio of mononuclear phagocytes to lymphocytes will influence the relationship between the supportive and the suppressive effect of mononuclear phagocytes. Secondly whether the macrophage mediated inhibition of lymphocyte proliferation stimulated by excess of antigen will be avoided by the more physiological presentation of small amounts of antigen by preincubated macrophages. A third question was how the supportive effect of *mononuclear phagocytes is related to the dif*ferentiation of monocytes to macrophages. The last question was whether the immuno-inductive function of mononuclear phagocytes is influenced by histocompatibility between macrophages and lymphocytes, as has been demonstrated in guinea pig systems

MATERIALS AND METHODS

Separation Techniques Venous blood drawn from healthy adults was defibrinated in Erlenmeyer flasks, using a glass stirrer (17) Mononuclear cells were separated from the blood by centrifugation on Isopaque/Ficoll, as described previously (15) The cells from each donor were employed in one experiment only

Macrophage cultures *The cells were suspended* in culture medium consisting of 80 per cent RPMI 1640 (Gibco Bio-Cult Glasgow Scotland) and 20 per cent pooled human AB serum supplemented by 0 1 mg l-glutamin per ml (later called complete culture medium) Gentamycin was added to the RPMI 1640 in a concentration of 40 mg/l. Macrophage cultures were prepared in plastic petri dishes or on glass coverslips placed in petri dishes, as described previously (15)

Lymphocyte cultures The lymphocytes were contaminated with different amounts of monocytes. Mononuclear blood cells, separated as described, contained 19 per cent monocytes (range 15–23 per cent) later designated Ly The non-adherent cells removed after a 90 min attachment period contained 4 per cent monocytes (range 2–7 per cent) designated Ly* In some experiments these cells were applied to petri dishes a second time, and the non-adherent cells were removed 90 min later These cells contained 0 5 per cent monocytes (range 0.3–0 8 per cent) and are termed Ly** *The monocyte contamination was evaluated by* counting adherent and non-adherent cells, amount*ing to a total of 500 cells in each culture in an* inverted phase contrast microscope. In each ex*periment, only one of the triplicate cultures* was evaluated in this way The values are the means of 10 experiments.

The lymphocytes were suspended in complete medium in a concentration of 10^6 cells per ml and 2.5 ml was added to each petri dish (Ø 50 mm) The dishes contained mononuclear phagocytes covering either the bottom of the dish or covering coverslips placed in the dish. The control dishes contained no precultured mononuclear phagocytes.

In some of the experiments the lymphocytes were cultured for two or three days in Kimax tubes before application to petri dishes. The tubes were centrifuged at 80 *g* for 10 min immediately after setting up the Kimax cultures

Induction of DNA synthesis in lymphocytes For stimulation with a soluble antigen, purified tuberculin (PPD) was used. In some experiments, PPD was added in a final concentration of 2 µg per ml *culture medium. In other experiments* mononuclear phagocytes were preincubated for one hour with culture medium containing 2 µg PPD per ml. The dishes were then washed four times with RPMI 1640 before lymphocytes were added. Lymphocytes were separated from persons who showed a strong positive tuberculin reaction, and are termed immune lymphocytes. In some of the experiments, lyophilized phytohaemagglutinin (PHA) from Gibco reconstituted with 10 ml destilled water was used in a quantity of 20 µl per ml culture medium. Both PPD and PHA was added *in optimal doses according to the dose-response effect described in a previous paper (15) DNA-synthesis* was induced by allogeneic lymphocytes in some *experiments.* Mixed lymphocyte cultures were prepared by mononuclear cells (Ly) from two individuals, making a final concentration of 5×10^5 cell per ml of each genotype.

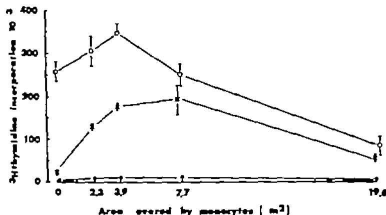


Fig. 2. ^{3}H thymidine incorporation in lymphocytes (Ly* containing 4 per cent adhesive cells) present to autologous monocytes precultured for three hours and covering different areas of the petri dishes. (O——O) PPD was added to a final concentration of 2 μg per ml culture medium (×——×) the monocytes were preincubated for one hour with medium containing 2 μg PPD per ml (●——●) non-stimulated control. The values are means ± SE of six experiments carried out in duplicate.

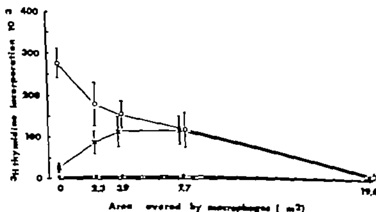


Fig. 3. ^{3}H thymidine incorporation in lymphocytes (Ly*) present to autologous macrophages precultured in vitro for 5–7 days (O——O) PPD was added to a final concentration of 2 μg per ml culture medium (n = 4) (×——×) the macrophages were preincubated for one hour with medium containing 2 μg PPD per ml (n = 6) (O——O) non-stimulated controls (n = 6). The values are means ± SE of experiments carried out in duplicate.

portive role of monocytes in induction of the secondary immune response was further elucidated by preincubating monocytes with antigen before immune lymphocytes were added. Autologous monocytes covering varying areas of the dishes were incubated for one hour with 2.5 ml complete medium containing 2 μg PPD per ml. After washing PPD-supplied and control dishes four times with 10 ml RPMI 1640 lymphocytes (Ly*) containing an average of 4 per cent adhesive cells were added. The PPD-preincubated monocytes showed a high capacity for inducing DNA synthesis in lymphocytes (Fig. 2). However when PPD was added directly to monocyte/lymphocyte cultures in a concentration of 2 μg PPD per ml, the ^{3}H thymidine incorporation exceeded the response in lymphocytes cultured with PPD-preincubated monocytes.

TABLE 3 *DNA Synthesis in Lymphocytes with Varying Degrees of Monocyte Contamination in Response to PHA and PPD Stimulation*

Lymphocyte isolation technique	^{3}H thymidine incorporation × 10^{-3} PHA	PPD
Mononuclear leucocytes isolated by Ficoll/Isopaque centrifugation (Ly)	302 ± 37	222 ± 10
Adherent cells removed by incubation in dish for 90 min (Ly*)	329 ± 20	227 ± 16
Repetition of the adherance procedure (Ly**)	388 ± 26	129 ± 17

The values are means ± SE of six experiments carried out in duplicate.

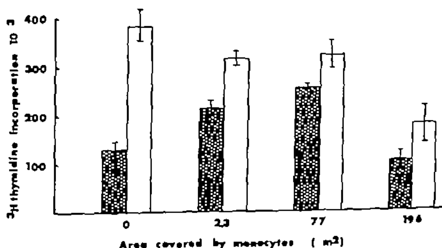


Fig 1 ^{3}H thymidine incorporation in lymphocytes (Ly** containing 0.5 per cent adhesive cells) when stimulated by PPD (■) or PHA (□) in the presence of autologous monocytes covering different areas of the culture dishes The columns and error bars indicate means ± SE of six experiments carried out in duplicate.

duced ^{3}H thymidine incorporation in lymphocyte cultures most depleted of adhesive cells (Ly**) was not reduced as compared to the incorporation in cultures with higher monocyte contamination (Ly* Ly) However the response in immune lymphocytes stimulated by PPD was significantly reduced in the less contaminated cultures (Table 3)

Effect of adding purified lymphocytes to precultured monocytes Lymphocytes were applied to petri dishes with autologous monocytes precultured in vitro for three hours. The monocytes covered a small coverslip (2.3 cm²) or two large coverslips (7 7 cm²) placed in the petri dish. In some cultures the monocytes covered the whole bottom of the petri dish (19 6 cm²) When purified immune lymphocytes and PPD were added to petri dishes with monocytes covering 2.3 cm² and 7 7 cm² of the area the reduced response was restored (Fig 1) When the monocytes covered the whole petri dish area (19 6 cm²) there was suppression of the response. Culturing of PHA stimulated purified lymphocytes in the presence of autologous monocytes covering 2.3 and 7 7 cm² of the dishes did not influence the thymidine incorporation significantly As in the PPD stimulated cultures, inhibition was seen when the monocytes covered the whole bottom of the dish.

Lymphocyte response to mononuclear phagocytes preincubated with PPD The sup-

cells. This lack of monocyte requirement in lectin-induced DNA synthesis is apparently in conflict with recent observations of other investigators. *Rosenstreich et al* (10) have demonstrated that a population of lymphocytes containing 0.3 per cent macrophages prepared from guinea pig lymph nodes did not respond to PHA or Con A. A population of murine thymocytes was also shown to require macrophages to respond to Con A (7). Human macrophages prepared from venous blood have been found to increase the response of purified lymphocytes to suboptimal PHA concentrations, but not to optimal doses (6). In our experiments optimal doses of both PHA and PPD were used (15). The results clearly indicate, therefore, that DNA synthesis in immune lymphocytes stimulated by PPD is more dependent on monocytes than PHA-induced DNA synthesis.

The presentation of antigen to lymphocytes by pretreated macrophages is a well-known phenomenon in animal systems (3, 5, 9). It was not surprising to find that human monocytes and macrophages had the same ability. A remarkable finding was that monocytes appeared to be more capable of retaining and presenting antigen than macrophages cultured in vitro for 5–7 days, in spite of the increase in cytoplasmic volume and membrane surface observed during in vitro culturing. The observations could be explained by the loss of some adherent cells during the preculturing period, cells that might even be able to synthesize DNA in response to antigen. However, this explanation is unlikely because washing twice before and four times after the antigen treatment removed all nonattached and probably most of the loosely attached cells from the monocyte cultures.

It seems clear from these results that human monocytes attached to a glass surface do not increase their ability to retain and present antigen when differentiating to macrophages, although the data presented do not justify a statement of an inverse relationship between the ability to present antigen and the differentiation to macrophages. It should be mentioned, however, that this apparent decrease in ability to retain and present antigen is parallelled by an increase in the ability of mononuclear phagocytes to inhibit DNA synthesis in stimulated lymphocytes, as shown in a previous paper (15).

Allogeneic macrophages preincubated with antigen stimulated DNA synthesis in immune lymphocytes less than autologous macrophages treated with antigen. This is in agreement with experiments of *Rosenthal & Shevach* (9, 12, 13) which indicate that effective presentation of antigen to lymphocytes by PPD-treated macrophages in a secondary immune response requires histocompatibility.

Culturing of PHA-stimulated lymphocytes in petri dishes gave a better response than culturing in Kimax tubes. The cause may be the crowding of cells at the bottom of the Kimax tubes. It is interesting to note that both PPD-stimulated cultures and mixed lymphocyte cultures gave the best response when the cells were cultured for half of the culture period in Kimax tubes before transfer to dishes. This can be explained by two competing conditions, the deleterious influence of culturing in Kimax tubes and the beneficial influence of close contact between lymphocytes and monocytes during induction of DNA synthesis.

It is obvious that the inhibitory effect on DNA synthesis in lymphocytes mediated by macrophages is a general phenomenon. We have shown previously that the response in mixed lymphocyte cultures and cultures stimulated by PHA and PPD in excess was inhibited by macrophages. When antigen is presented by pretreated macrophages, the stimulation of lymphocytes increases with increasing ratio of macrophages to lymphocytes up to a certain point. When exceeding this ratio, DNA synthesis is suppressed, thus indicating than even lymphocytes stimulated by macrophages treated with antigen are subject to the same inhibitory influence.

The technical assistance of Mrs. *A. Renarx* and Mrs. *B. Lippe* is gratefully acknowledged. I am also indebted to Professor *J. Lamvik* for discussion and help during the course of this study and in

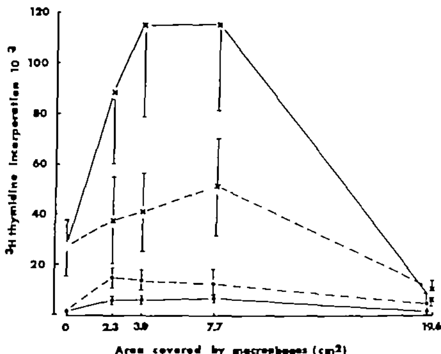


Fig 4 ³H thymidine incorporation in lymphocytes (Ly⁺) present to autologous (——) or allogeneic (– – –) macrophages, precultured in vitro for 5–7 days. (×——× ×– – –×) the macrophages were preincubated with PPD (●——● ●– – –●) controls not preincubated with PPD The values are means ± SE of six experiments carried out in duplicate.

The response was related to the size of the area covered by monocytes. The supportive influence turned to suppression when the monocyte-covered area, which is an expression of the ratio of monocytes to lymphocytes, increased beyond a certain size.

Autologous macrophages precultured in vitro for 5–7 days before incubation with PPD and exposure to immune lymphocytes also induced ³H thymidine incorporation but the response to macrophages was smaller than the response to monocytes preincubated with PPD (Fig 2 Fig 3) When PPD was added directly to the culture dishes the macrophages had a considerable suppressive effect, even in a small number The suppression increased with an increasing ratio of macrophages to lymphocytes (Fig 3) The response to macrophages preincubated with PPD was less influenced by this inhibitory effect, but it did not exceed the response to PPD added directly to the culture dishes Allogeneic macrophages incubated with PPD showed on the average, less ability to induce ³H thymidine incorporation in immune lymphocytes than autologous macrophages treated in the same way (Fig. 4) The ³H thymidine incorporation in the controls without PPD was generally low but it was notably higher in an allogeneic system than in an autologous system.

DISCUSSION

The reduced response to a soluble antigen (PPD) observed in immune lymphocytes depleted of adhesive cells is in agreement with observations made in a guinea pig system (11) Furthermore, the macrophage dependence of antigen induced in vitro lymphocyte proliferation has been observed in a number of mammalian species such as rats and rabbits (8) The restoration of the reduced response when the cells were cultured in the presence of autologous adherent cells confirms the result. When stimulated by PHA, purified lymphocytes responded as well as lymphocytes contaminated with adhesive

Acta path. microbiol. scand. Sect. C, 85: 389–394, 1977

AGGLUTININS TO RABBIT ERYTHROCYTES IN EXTRACTS OF HUMAN MALIGNANT TISSUES

FINN WESENBERG and OLAV TÖNDER

Broegelmann Research Laboratory for Microbiology
and Department of Microbiology, The Gade Institute, University of Bergen, Bergen, Norway

Wesenberg, F. & Tönder, O. Agglutinins to rabbit erythrocytes in extracts of human malignant tissues. Acta path. microbiol. scand. Sect. C, 85: 389–394, 1977.

Agglutinins to rabbit erythrocytes in extracts from human malignant tissues were identified as the naturally occurring IgG antibodies in human serum to rabbit erythrocytes. This was revealed by agglutination, absorption, antiglobulin and inhibition tests, immunization of rabbits and immunochemical analyses. The agglutinins can therefore be used as convenient markers for both extracellular immunoglobulin and unspecifically bound immunoglobulin in tumour tissues. Apparently the extracts also contained small amounts of IgA antibodies to rabbit erythrocytes.

Key words: Human tumours; tissue extract; haemagglutinins.

F. Wesenberg, Broegelmann Research Laboratory for Microbiology, N-5016 Haukeland sykehus, Bergen, Norway

Received 24.ii.77. Accepted 30.vi.77

In 1908 *Landsteiner* (4) described an agglutinin to rabbit erythrocytes in saline extracts of murine sarcoma and carcinoma. The agglutinin was not present in extracts of normal murine tissues.

Forty years later *Salaman* (7) detected agglutinins to erythrocytes of various species in saline extracts of murine tumours. These agglutinins were labile, i.e. they lost half their activity when kept for 1 h at ambient temperature (approx. 20° C) and lost all activity after 24 h. The activity could be preserved, or lost activity regenerated by using 2,3-dimercapto propanol (British Anti-Lewisite (BAL) 0.01 M). Low activity was found in extracts of normal tissue.

Humphrey (2) further distinguished between haemadsorption and agglutination when tissue sections of murine sarcoma were tested against rabbit erythrocytes. None of these authors identified the agglutinins. However *Zwillenberg & Hallauer* (11) described an agglutinin to human 0 erythrocytes in human tissue culture and they found it was probably a virus-like particle.

During previous investigations (9) on haemadsorption to tissue sections of erythrocytes of various species, we observed that rabbit erythrocytes agglutinated on sections of most human tumours when using a closed chamber technique (unpublished). However the erythrocytes were either not bound or were only loosely bound to the tissue. With reference to the literature cited, the purpose of this study was to characterize the aggluti-

the preparation of the manuscript. This work was supported by grants from the *Norwegian Research Council for Science and the Humanities* the *Norwegian Cancer Society* and the *Norwegian Society for Fighting Cancer*

The author is a research fellow of the *Norwegian Research Council for Science and the Humanities*

REFERENCES

1 *Askonas B. A & Roelants G E.*. Macrophages bearing hapten-carrier molecules as foci inducers for T and B lymphocyte interaction. Eur J Immunol. *4* 1–4 1974

2 *Calderon J & Unanue E R* Two biological activities regulating cell proliferation found in cultures of peritoneal exudate cells. Nature *253* 359–361 1975

3 *Calderon J Kiely J M Lefko J L & Unanue E R* The modulation of lymphocyte functions by molecules secreted by macrophages, I Description and partial biochemical analysis. J Exp. Med. *142* 151–164 1975

4 *Diamantstein T & Ulmer A* Two distinct lymphocytestimulating soluble factors (LAF) released from murine peritoneal cells. I Cellular source and the effect of cGMP on their release. Immunol. *30* 741–747 1976

5 *Katz D H & Unanue E R.* Critical role of determinant presentation in the induction of specific responses in immuno-competent lymphocytes. J Exp Med. *137* 967–990 1973

6 *Mc Combs C Michalski J P & Talal N.*. Cellular interactions in lymphocyte proliferation Effect of syngeneic and xenogeneic macrophages. Cell Immunol *23* 283–296 1976.

7 *Mills G Monticone V & Paetkau V.*. The role of macrophages in thymocyte mitogenesis. J Immunol *117* 1325–1330 1976

8. *Oppenheim J J Elfenbein G J & Rosenstreich D L.* The role of macrophages in B and T lymphocyte transformation. In van *Furth R.* (Ed.): Mononuclear phagocytes in immunity infection and pathology Blackwell Scientific Publication, Oxford, 1975 p. 794–812.

9 *Rosenthal A S & Shevach E. M.*. Function of macrophages in antigen recognition by guinea pig T lymphocytes. I Requirement for histocompatible macrophages and lymphocytes. J Exp Med. *138* 1194–1212 1973

10 *Rosenstreich D L Farrar J J & Dougherty S* Absolute macrophage dependency of T lymphocyte activation by mitogens. J Immunol. *116* 131–139 1976

11 *Seeger R C & Oppenheim J J.*. Synergistic interaction of macrophages and lymphocytes in antigen-induced transformation of lymphocytes. J Exp Med. *132* 44–65 1970

12. *Shevach E. M & Rosenthal A S.*. Function of macrophages in antigen recognition by guinea pig T lymphocytes. II Role of the macrophage in the regulation of genetic control of the immune response J Exp. Med. *138* 1213–1229 1973

13 *Shevach E. M* The function of macrophages in antigen recognition by guinea pig T lymphocytes. III Genetic analysis of the antigens mediating macrophage-T lymphocyte interaction. J Immunol. *116* 1482–1489 1976.

14 *Unanue E R. & Calderon J.*. Evaluation of the role of macrophages in immune induction Fed. Proc. *34* 1737–1742, 1975

15 *Unsgaard G & Lamvik J* Inhibitory effect of human mononuclear phagocytes on DNA synthesis in stimulated lymphocytes. Acta path. microbiol. scand. Sect. C, *85* 373–380 1977

16 *Ulken K E. & Odegaard A* Phagocytosis of heat killed radiolabelled *Candida albicans* by human blood monocytes cultured in vitro. Acta path. microbiol, scand. Sect. B, *82* 235–244 1974

17 *Odegaard A Ulken K. E. & Lamvik, J* Structural and functional properties of blood monocytes cultured in vitro. Acta path. microbiol. scand. Sect. B, *82* 223–234 1974

ml were added to each tube. The mixtures were incubated at 37 C for 30 min and 0.1 ml of a 0.5 per cent suspension of rabbit erythrocytes was added. The tubes were left at ambient temperature for 15 min and the degree of agglutination was recorded as described above. The titre of inhibition was defined as the highest dilution of antiserum which showed complete inhibition of agglutination.

Precipitation

The double diffusion test in agar was performed as described by *Ouchterlony* (6). One per cent agar in PBS was used.

Agglutination on Tissue Sections

Technique with closed chambers. Cryostat sections (6 μm thick) of frozen tissue on large cover glasses were incubated at ambient temperature with 0.5 per cent of a suspension of rabbit erythrocytes in PBS. Microculture slides with single concavity were used, which allowed the erythrocytes to settle onto the tissue on the cover glass (9). After 30 min the slides were turned cover glass up. The preparations were immediately examined under microscope and the degree of agglutination was recorded as follows: 3+ Large clumps with few single erythrocytes. 2+ A mixture of large and small clumps and many single erythrocytes. 1+ Some small clumps (three to five erythrocytes) but mainly single erythrocytes. The slides were then left for the sedimentation of the erythrocytes. The haemadsorption to tissue was recorded microscopically when the glass around the tissue was free of erythrocytes. The larger agglutinates could be observed on the bottom of the concavity.

Technique with open glass slides. One drop of a 0.5 per cent suspension of rabbit erythrocytes, enough to cover the cryostat section on the slide, was applied and the preparation was left for 5 min at ambient temperature. The slide was then gently tilted and the degree of agglutination of erythrocytes was recorded macroscopically (2+, 1+, —).

Separation Techniques

A Sephadex G-150 column was prepared with a void volume of 140 ml, total volume of 370 ml, and with an inner diameter of 2.5 cm, equilibrated with PBS. The flow rate was approx. 0.5 ml/min and each fraction was approx. 6 ml.

Electrophoresis in agar and immunoelectrophoresis were performed as described previously (8).

Various Reagents

Ethanol, acetone, formaldehyde 4 and 10 per cent, NaCl solutions and phosphate, glycine and acetat buffers with pH ranging from 2.0 to 11.0 were standard reagents.

RESULTS

Agglutination on Tissue Sections

When tissue sections were tested against rabbit erythrocytes using the closed chamber technique, agglutinates ranging from 3+ to 1+ were observed. Most of the tissues gave a 2+ reaction. The agglutinates were weakly bound to the tissue but after 1 h no agglutinates were attached to the sections. Although the degree of agglutination varied among the malignant tissue, all possessed the agglutinating activity. Furthermore, sections of all normal tissues showed similar activity and behaviour. Using the technique with open glass slides, we observed the agglutinates leaving the tissue sections just as the slides were tilted. Most of the tissues gave a 2+ reaction.

These results indicated that the agglutination of rabbit erythrocytes was due to soluble or extractable factors in the tissues.

This conclusion was further supported by the results obtained from the following experiments. Tissue sections were washed for 10 min by leaving the cover glasses in beakers filled with PBS. In subsequent tests with rabbit erythrocytes all agglutinating activity was lost. Even after a short washing of 30 s the activity was considerably reduced.

Likewise, tissue sections were left in beakers filled with acetone, ethanol, formaldehyde solutions, various salt concentrations and various buffer solutions with pH ranging from 2.0 to 11.0. The agglutinating activity could not be preserved or fixed in the tissue sections.

Extracts

Extracts from malignant and normal tissues studied in agar and immunoelectrophoresis gave serum-like patterns, as expected.

Representative results obtained with extracts in direct agglutination and antiglobulin tests are shown in Table 1. The titres of direct agglutination ranged from one to eight, and increased from four to eight times in the

nating factor and investigate whether it was present only in malignant tissue

MATERIALS AND METHODS

Tissues

Specimens from 18 human malignant tumours and normal liver lung kidney muscle lymph node and spleen were kindly provided by the Department of Pathology University of Bergen. Tissue not used immediately was stored at –25 C Cryostat sections of tissue were prepared and handled as described previously (9) Histological investigation of tumours was routinely performed at the Department of Pathology

Extracts

Tissue was homogenized in phosphate buffered saline (PBS) pH 7 2 (four times wet weight) in a Sorval Omni-Mixer in an ice water bath for 5 min. The homogenate was centrifuged at 10 000 × *g* for 20 min at 5 C. The supernatant beneath the lipid layer (extract) and the tissue sediment were stored at –25 C When necessary the extract was concentrated in collodium bags using negative pressure (3) For some experiments extracts were digested with pepsin (1)

Sera

Antiserum to whole human serum was produced as previously (10) and antisera to human IgG and IgM were produced and tested for specificity as described by *Larsen* & *Tönder* (5) Antisera to IgG and IgM were also purchased from Behringwerke AG (Marburg Lahn West Germany) Antisera to serum IgA were purchased from Dakopatts A/S Copenhagen.

Antiserum to rabbit erythrocytes agglutinated by an extract of a renal carcinoma (abbr antiserum to tumour haemagglutinins) was produced by immunization of a rabbit with its own erythrocytes coated with two agglutinating units of the extract (see below) Coating of the erythrocytes was performed by suspending 0 4 ml of packed erythrocytes in the correct volume of undiluted extract (If the agglutination titre of the extract was 4 the volume needed for sensitization was 40 ml) After 15 min the erythrocytes were packed by centrifugation and thereafter washed twice in 100 ml of PBS The coated erythrocytes were suspended to 2 ml (20 per cent suspension) and injected subcutaneously on the back of the rabbit. The immunization schedule consisted of weekly injections for six weeks. Small blood samples were drawn from the ear veins before each injection, and a larger sample one week after the last injection. Booster doses were given one week before later bleedings.

A high-titred rheumatoid serum was used as human anti globulin (rheumatoid factor)

The anti-globulin titre of a serum was defined as the reciprocal value of the highest dilution which agglutinated rabbit erythrocytes sensitized by one half agglutinating unit of human IgG

A pool of human sera from 20 blood donors (PHS) was used as normal serum. Isolated human IgG was purchased from AB Kabi, Stockholm. The preparation contained less than 10 per cent IgA.

Erythrocytes

Erythrocytes of various animal species were obtained from whole blood collected in Alsevers solution. Human blood was collected in sterile acid-citrate-dextrose solution. Before use the erythrocytes were washed four times in 6–10 volumes of PBS They were finally packed at 1,000 × *g* for 10 min (100 per cent)

Haemagglutination in Tubes

To twofold dilutions of extracts or serum in 0 1 ml volumes of PBS was added 0 1 ml of a 0.5 per cent suspension of rabbit erythrocytes. The mixtures were left for 15 min at ambient temperature. The tubes were centrifuged at 1,000 × *g* for 30 s, the erythrocytes were gently resuspended and agglutination was recorded macroscopically The titre of agglutination was defined as the reciprocal value of the highest dilution showing agglutination One agglutinating unit was defined as the amount of the highest dilution of extract or serum which agglutinated an equal amount of a 0.5 per cent suspension of erythrocytes.

After the direct agglutination was recorded, the erythrocytes in each tube were washed twice in 2 ml of PBS The erythrocytes were then resuspended in 0 1 ml of either antisera to human immunoglobulins or antiserum to tumour haemagglutinins diluted to 1/40th the anti-globulin titre dilution The tubes were left at ambient temperature for 5 min centrifuged at 1 000 × *g* for 30 s and the agglutination was recorded as described above

Absorption with Erythrocytes

To 0 3 ml of tumour extract was added 0 1 ml of packed erythrocytes. The suspension was left at ambient temperature for 30 min, and was then centrifuged at 1,000 × *g* for 10 min. The supernatant was tested for agglutinins to rabbit erythrocytes

Inhibition of Haemagglutination

Twofold dilutions of antisera to human serum or to Igs were prepared in 0 1 ml volumes of PBS Two agglutinating units of extracts in 0.1

guinea-pig. None removed the activity against rabbit erythrocytes.

Inhibition Studies with Anti Ig

Although it is difficult to measure inhibitory activity in an agglutination system with an antiglobulin serum because of the potentiating effect of the antiglobulin, inhibition studies were performed with antisera to various immunoglobulin classes. With all extracts tested, antserum to human serum, anti-IgG and antiserum to tumour haemagglutinins showed inhibition up to a dilution of 1 in 8. No inhibition was obtained using antisera to the other Ig classes. This indicated that IgG constitutes the larger part of the agglutinins to rabbit erythrocytes.

Fractionation of Extracts

Extracts of malignant tissues were concentrated eight times and 1 ml was fractionated on a Sephadex G-150 column. The fractions were concentrated × 50 and were tested for the presence of serum proteins and agglutinins to rabbit erythrocytes. Agglutinating activity was convincingly found in the fractions containing IgG and IgA.

DISCUSSION

We have described agglutinins to rabbit erythrocytes present in human tissues. These were demonstrated by incubating cryostat sections of tissue with the erythrocytes which were agglutinated on the sections. The agglutinates did not attach to the tissue. The activity disappeared when the sections were washed 10 min in PBS, and fixatives did not preserve it. Consequently the agglutinins are soluble and extractable from the tissue.

All evidence is in favour of the agglutinins being IgG antibodies. In extracts of the tissues the agglutinins were found in the fractions containing IgG; anti-IgG including rheumatoid factor augmented the agglutination, and immunization with erythrocytes agglutinated by these agglutinins stimulated to the production of anti-IgG. Further pepsin-digested extracts agglutinated similarly but rheumatoid factor did not influence this agglutination. This accords with some properties of F(ab) fragments of IgG.

Since the agglutinins were inhibited by antisera to IgG and antiserum to tumour haemagglutinins and not inhibited by antisera to the other Ig classes, these agglutinins to rabbit erythrocytes are probably identical to the corresponding naturally occurring IgG antibodies found in human sera (10). This was further supported by the results of absorptions with human and animal erythrocytes.

In all investigations performed we found no essential difference between extracts of malignant and normal tissue. The agglutinins are therefore not associated with human malignant tissue. Most likely they would be found in all extracellular fluids in the body or unspecifically bound to various cells. In this respect the agglutinins must be different from the agglutinin to rabbit erythrocytes in extracts from murine malignant tissues described by *Landsteiner* (4) and *Salaman* (7). Their agglutinin was unstable, and apparently characteristic for malignant tissue, since very low activity was found in extracts of normal tissue.

We have not made sufficient studies to quantify the IgG in extracts of malignant and normal tissues, but apparently the concentrations are very similar in the two types of tissues.

The agglutinins to rabbit erythrocytes would therefore serve as an indicator of the IgG in the ratio titre of antibodies to concentration of IgG. Preliminary results indicate that this ratio is similar with extracts of malignant tissue and extracts of corresponding normal tissue. The IgG antibodies to rabbit erythrocytes can accordingly be used as a marker antibody in studies of immunoglobulins present in tissues.

These marker antibodies will be particularly valuable in the characterization of eluates of malignant and normal tissues. The question of whether the immunoglobulins present in eluates from malignant tissues re-

antiglobulin test with antiserum to IgG A similar antiglobulin effect was obtained with antiserum to human serum, antiserum to tumour haemagglutinins and with rheumatoid factor Antisera to IgM and IgA did not influence the titres. Extracts of malignant and normal tissues behaved similarly Pepsin digested extracts also agglutinated rabbit erythrocytes, but rheumatoid factor did not potentiate this agglutination. The results indicated that at least IgG participated in the agglutination of rabbit erythrocytes.

TABLE 1 *Agglutination of Rabbit Erythrocytes by Extracts of Human Malignant and Normal Tissues. Titres in Direct Agglutination and Antiglobulin Test with Anti-IgG*

Extracts of	Direct agglutination	Antiglobulin test
Carcinoma of		
kidney	4	16
colon	8	64
breast	2	8
bronchus	2	8
lung	1	8
Normal tissue		
kidney	4	16
liver	4	16
muscle	4	32

This was further supported by the results of double diffusion in agar (Fig 1) Extracts of malignant and normal tissues showed one marked identity line with human serum and with isolated IgG when tested against the antiserum to tumour haemagglutinins. In addition two weaker lines appeared with serum and the extracts. One of these lines represented IgA as shown by the results of immunoelectrophoresis (Fig 2) That the weak IgA line was not produced by antibodies to light chains was verified by the results of double diffusion in agar A clear identity line appeared using antisera to tumour haemagglutinins and to α-chains against PHS These results indicated that both IgG and IgA were present on the erythrocytes used for immunization. The anti

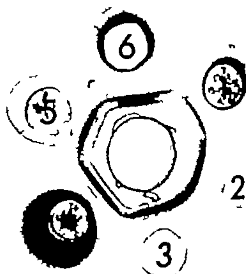


Fig 1 Double diffusion in agar Center well antiserum to tumour haemagglutinins. Well 1 extract from normal muscle. Then clockwise isolated IgG, pooled human serum (PHS) and extracts from 3 different malignant tumours.

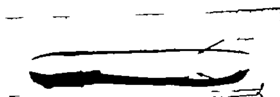


Fig 2 Immunoelectrophoresis of human serum. Upper trough antiserum to tumour haemagglutinins. Lower trough polyvalent antiserum to immunoglobulins. The troughs were filled twice. The IgA lines are indicated by an arrow

globulin titre of the antiserum to tumour haemagglutinins was 2.048 in the test using rabbit erythrocytes sensitized by one half agglutinating unit of IgG

The extracts lost no agglutinating activity after incubation at 37 C for 60 min, and showed no drop in titres after 14 days at ambient temperature or at —25° C for six months.

Some extracts were also absorbed with human AB D positive erythrocytes and erythrocytes from other species ox, sheep mouse and

Acta path. microbiol. scand. Sect. C, 85: 395–397 1977

BRIEF REPORTS

B- AND T-CELLS AND Ig-CONTAINING BLAST CELLS IN HEALTHY CHILDREN

Poul Aabel Østergaard & Jan Eriksen

Department of Clinical Chemistry Aalborg Hospital South, and
Blood Bank and Pathological Institute Aalborg Hospital North,
Aalborg, Denmark

Østergaard, P Aa. & Eriksen, J B- and T-cells and Ig-containing blast cells in healthy children. Acta path. microbiol. scand. Sect. C, 85 395–397 1977

Studies on rosette-forming cells and IgG IgA, IgM and IgE-bearing lymphocytes were carried out in 70 healthy children aged 5 to 15 years. Furthermore, in 20 of these children, the percentages of IgG IgA, IgM and IgE-containing blast cells after pokeweed mitogen stimulation of peripheral lymphocytes were evaluated N differences in the percentages of rosette forming cells, or in Ig-bearing and Ig-containing cells were found when three different age-groups were compared.

Key words: Rosette-forming cells Ig-bearing lymphocytes Ig-containing blast cells healthy children.

P Aa. Østergaard, Department of Pediatrics, Aalborg Hospital North,
DK 9100 Aalborg, Denmark.

Received 30.ii 77 Accepted 1 viii 77

The value of enumeration of rosett-forming cells (E RFC) in the evaluation of T-cell subpopulations is generally accepted (*Fu & Kunkel* 1974) Furthermore, in the investigation of different immunodeficiency disorders, the estimation f immunoglobulin (Ig-) bearing and Ig-containing cells a aluable and helpful tool. The aim of the present publication is to present the results of studies of E-RFC and Ig-bearing peripheral lymphocytes in 70 healthy children In addition, the percentage of Ig-containing blast cells after PWM stimulation of peripheral lymphocyte in 20 of these children was evaluated.

Material and Methods

The series comprises 70 healthy school and preschool hildren, aged 5 t 15 years They were d ided into three groups according to age 1) 25 children aged 5 to 8 years, 2) 22 children aged 9 to 11 years and 3) 23 children aged 12 to 13 years.

Isolation of the lymphocytes, the performance of the E-rosett test and the studies on Ig-bearing and Ig-containing cells ha been reported elsewhere (*Østergaard* 1977) In brief peripheral lymphocytes were isolated on a Ficoll-Isopaque preparation according to the method of *Bøyum* (1967) The studies of *E-rosette forming* cells (E-RFC) were *performed* by the method of *Jondal et al* (1972) The sheep red blood cells (SRBC) were pretreated with papain (Merck) in order to increase the number of E-RFC (*Jondal* 1976) Lymphocytes with *three* or more SRBC attached to the surface were counted as rosettes

For the evaluation of Ig-bearing lymphocytes, the procedure used was essentially that of *Winchester & F* (1976) The FITC-conjugated IgG IgA and IgM antisera used were purchased from Dacopatts, Copenhagen, nd the conjugated IgE antiserum was obtained from Behringwerke AG Germany The lymphocytes were preincubated with latex at 37 C for 30 minutes in order to label contaminating monocytes and avoid capping of the F fragments of IgG (*Lobo et al.* 1975) before

present specific antibodies or unspecifically bound immunoglobulins, for example binding to the Fc receptors, is not solved at present. Studies are in progress utilizing this principle.

In addition to IgG small amounts of IgA were apparently attached to the erythrocytes as revealed by the immunization experiments. Whether this IgA acts as an antibody to the erythrocytes was not clarified. However the presence of such antibodies in tissue extracts corresponds well to their presence in serum (10) Possibly these IgA antibodies to rabbit erythrocytes also can be utilized as markers in studies on immunoglobulins associated with malignant tumours.

Dr *F Wesenberg* is a fellow of the Norwegian Research Council for Science and the Humanities.

REFERENCES

1 *Christie K E., Solberg C O Larsen B Grov A & Tönder O* Influence of IgG F(ab)$_2$ and IgM on the phagocytic and bactericidal activities of human neutrophil granulocytes. Acta path. microbiol scand. Sect. C, *84* 119–123 1976.

2. *Humphrey L. J* Studies on hemadsorption by murine tumours. Ph. D Thesis State University of New York. Buffalo 1967

3 *Kabat E A & Mayer M M.* Experimental Immunochemistry 2 ed Charles C. Thomas, Springfield, Ill. 1964 p 729

4 *Landsteiner K.* Bemerkungen zur Kenntnis der Übertragbaren tierischen Tumoren. Wien. klin. Wschr 21 1549–1550 1908.

5 *Larsen B & Tönder O* Preparation of anti human-γ-globulin sera not containing human serum proteins. Vox Sang *16* 69–72, 1969

6 *Ouchterlony Ö* Diffusion-in-gel methods for immunological analyses II Progr Allergy 6 30–154 1962

7 *Salaman M H.* Haemagglutination by extracts of tumours and of some normal tissue. Brit. J Cancer 2 253–267 1948.

8 *Thunold S Tönder O & Larsen, O.* Immunoglobulins in eluates of malignant human tumours. Acta path. microbiol scand. Sect. A, Suppl 236 97–100 1973

9 *Tönder O Milgrom F & Witebsky E.* Mixed agglutination with tissue sections. J exp. Med. *119* 265–274 1964

10 *Tönder O Natvig J B & Matre R* Antibodies in human sera to rabbit erythrocytes. Immunology *12* 629–637 1967

11 *Zwillenberg, L. O & Hallauer C* An unidentified hemagglutinin from human tumor tissue cultures. Arch. ges. Virusforsch. *12* 393–403 1962

in a few adults by *Waldman et al.* (1974) who found that an average of 7.9 per cent of the cells in the PWM stimulated cultures stained for IgM while 3.6 per cent were positive for IgA, and 5 per cent for IgG. The lower percentages of Ig containing cells in the present study may be due to differences in techniques used, in the age of the subjects studied, or in the antisera used.

The study was supported by *the Danish Medical Research Council* (Grant no 512 7178) and by *Det Lægevidenskabelige Forskningsfond for Nordjyllands Amt*

References: Broom, B. C D De La Concha E. G Webster A D B., Janossi G J & Asherson G. L: Clin exp. Immunol. *23* 545–549 1976 — *Buckley R. H* Birth Defects. Orig Art. Ser *11* 134–142, 1975 — *Böyum A* Scand J Clin. Lab. Invest. *21* 77–89 1967 — *Cambell, A C Waller C, Wood J Aynsley-Green A & Y I* Clin. exp. Immunol. *18* 469–475 1974 — *Fleischer T A Luckasen J R., Sabad A., Gertz P C & Kersey J H* Pediatrics *55* 162–165 1975 — *F S M & Kunkel H G* J exp. Med. *140* 895–899 1974 — *Jondal M Holm G & Wigzell H.* J exp Med. *136* 207–214 1972. — *Jondal M.* Scand. J Immunol. *5* suppl. 5 69–76, 1976 — *Lawton A R. Royal S A., Self K. S & Cooper M D* J Lab. Clin. Med. *80* 26–33 1972. — *Lobo P I Westervelt F B. & Horwitz, D A* J Immunol. *114* 116–119 1975 — *P pys, M B., Salegua-Guidett C., Mirjah D D Wansbrough-Jones H H & Dash A. C.:* Clin. exp. Immunol. *26* 91–94 1976. — *S hoeph E. & Boehringer D.* J Derm. *1* 133–144 1974 — *Waldman T A., Brod S Blaese R. M., D rm M., Blackman M & Strober W* Lancet *II* 609–613 1974 — *Winchester R J & F S M* Scand. J Immunol. *5* suppl. 5 77–89 1976. — *Winchester R. J., F S M., Hoffman T & Kunkel H G.* J Immunol. *114* 1210–1212, 1975 — *Ostergaard P Aa. & Blom M* (unpublished results) — *Ostergaard P Aa* Acta path. microbiol. scand. Sect. C, in press.

staining Incubation with antisera was carried out at 4 C The percentage of cells with a bright fluorescing semicircular or surrounding ring was determined

The studies of intracellular Ig-synthesis were performed by the method of *Waldmann et al.* (1974) and *Broom et al.* (1976) with minor modifications. Lymphocytes from 20 healthy children were cultured in RPMI 1640 (Biocult, Glasgow) supplemented with 10 per cent autologous serum for seven days at 37 C in a moistened 5 per cent CO_2 atmosphere in the presence or absence of 10 microlitre pokeweed mitogen (PWM MEDA, Copenhagen) per 0 25 ml lymphocyte suspension (1×10^6 cells per ml) On day 4 the cultures were supplemented with 0 1 ml fresh medium with serum On the termination of the culture period, blast cell proliferation was assessed under an inverted microscope, and 0 075 ml of the cell pellet was incubated with 0 025 ml of the FITC-conjugated IgG- IgA IgM and IgE-antisera. The percentage of blast cells with bright fluorescence of the cytoplasma was determined in a Leitz Orthoplan fluorescence microscope.

All the children had the levels of IgG IgA and IgM in serum considered normal for healthy children of that age (*Østergaard & Blom* 1977)

Statistical evaluation of the results was performed by the Mann-Whitney test A significance level of 5 per cent was chosen.

Results and Discussion

The results of the lymphocyte subpopulation studies are shown in Table 1 The results of the E-RFC studies in the various age-groups showed no differences between the medians ($p > 0 1$) The proportion of E-RFC in the present study is somewhat higher than in other studies of E-RFC in children (*Campbell et al.* 1974 *Fletcher et al.* 1975) This may be due to pretreatment of the SRBC with papain used in our study which is known to increase the percentage of E-RFC (*Jondal* 1976)

As regards the results of the studies of the Ig bearing cells (Table 1) the median values obtained showed no differences between the various age-groups ($p > 0 1$) The median percentages of IgG-bearing lymphocytes in the present study are considerable lower than those obtained by others (*Lawton et al.* 1972 *Buckley* 1975) However these authors did not pretreat the lymphocytes at 37 C before staining which is known to inhibit the binding of a special subpopulation of lymphocytes with IgG Fc receptors to the SRBC (*Lobo et al.* 1975) Pretreatment at 37 C or the use of $F(ab)_2$ reagents instead of whole antibodies to IgG results in a lower percentage of lymphocytes staining for IgG (*Winchester et al.* 1975) Furthermore, in the present study the results obtained for membrane staining with both IgG and IgA and IgM are in agreement with recent studies in adults performed by *Pepys et al.* (1976) As regards membrane-bound IgE, the median of 1 0 per cent in the present study is somewhat lower than the value reported by *Schoeph & Boehringer* (1974)

TABLE 1 *Ranges and Medians of Rosette-forming Cells (E RFC) and of Ig-bearing lymphocytes in the Various Age Groups*

Subjects	E RFC (%)	Ig-bearing lymphocytes (%) IgG	IgA	IgM	IgE
5–8 Years					
Range	55–82	0–4	0–4	7–16	0–3
Median	70 0	2.0	1 0	10.5	1 0
9–11 Years					
Range	61–80	0–4	0–5	5–15	0–4
Median	72.5	2.5	1.5	11 0	1.5
12–15 Years					
Range	52–81	0–5	0–3	4–16	0–4
Median	72 0	2.5	1 0	9.5	1.5

The results concerning Ig-containing blast cells after PWM stimulation of peripheral lymphocytes are shown in Table 2 Very little information is available regarding proliferation studies of peripheral lymphocytes from healthy children with subsequent counting of Ig-containing blast cells. Cells containing IgG IgA and IgM were studied

TABLE 2. *Ranges and Medians of Ig-bearing Lymphocytes and of Ig-containing Blast Cells after PWM stimulation of Peripheral Lymphocytes in 20 Healthy Children*

Subjects (6–11 Years)	Ig-bearing lymphocytes (%) IgG	IgA	IgM	IgE	Ig-containing cells (%) IgG	IgA	IgM	IgE
Range	0–4	0–5	7–15	0–5	1–4	0–4	5–10	0–5
Median	2 0	1.0	10.5	1.5	2.5	1.5	8.5	1.5

added at room temperature during 2.5 min. Finally the cells were centrifuged at 100 *g* for 5 min and resuspended. Cultures were performed in *round-bottom microplates* (*Linbro Chemicals, New Haven, Conn.*) in 150 μl RPMI 1640 with 5 per cent FRS and 100 IU Penicillin and 100 μg Streptomycin per ml. Cell concentrations were used which gave a maximal response without having reached a plateau value. Triplicate cultures of 2×10^5 cells were stimulated with 0.1, 0.3 and 0.9 per cent PHA (Phytohemagglutinin-P, Difco, Detroit, Michigan). The results obtained with the PHA concentration which gave the maximal response (almost exclusively the 0.3 per cent PHA samples) were used, and values obtained in unstimulated cultures were subtracted. Six-duplicate cultures of 1×10^5 Ho cells were stimulated with 1×10^5 F cells. Values obtained from the same number of unstimulated cells were subtracted. 1.25 μCi (Methyl-^{3}H) thymidine (The Radiochemical Centre, Amersham, England) specific activity 5 Ci/mmol, was added for the last 4 h before harvesting with a Skatron Multiple Cell Culture Harvester. PHA-stimulated cultures were terminated at 48 h and MLR cultures at 96 h. Radioactivity was measured by a Beckman Soft Beta Liquid Scintillation Counter. The popliteal lymph node assay described by *Ford et al.* was used for estimation of graft-versus-host (GVH) activity (2). Three graded cell doses of fresh or frozen-thawed Hooded cells were injected into the foot-pads of F hybrid rats in a volume of 0.1 ml. Four to six foot pads were injected with each dose. Seven days later the draining popliteal nodes were removed and weighed.

Results and Discussion

As shown in Table 1, response to PHA and fresh semiallogeneic cells were lower in frozen-thawed than in fresh cell cultures (68–87 per cent and 75–87 per cent that of fresh cells, respectively). As stimulators in the MLR, frozen-thawed cells induced 70–80 per cent ^{3}H-thymidine uptake compared to fresh cells, and when used as both partners in the MLR, the reaction was even more diminished: 64–70 per cent that of corresponding fresh cells. The results from one GVH experiment are shown in Fig. 1. The ability to produce GVH node enlargement could be derived by comparing doses of cells required to produce a given popliteal node weight (11). The GVH activity of frozen-thawed cells was thus about 75 per cent that of corresponding fresh cells. With a different freezing protocol, only about 40 per cent post-thaw activity of PHA stimulated lymph node cells compared with corresponding fresh cells has been obtained previously (4). The method of slow addition and dilution of the cryoprotectant at room temperature to allow for osmotic equilibrium has proved to be essential for maximum recovery of functional lymphocytes (7, 8, 9). The present findings on PHA

TABLE 1. *Response of Fresh and Frozen-thawed Hooded Lymphocytes to PHA and Fresh and Frozen-thawed Semi-allogeneic F (Hooded × AGUS) Lymphocytes*

Exp. No.	Stimulant	^{3}H-thymidine incorporation (cpm ± 1 SD)	
		Fresh Ho cells	Frozen-thawed Ho cells
1	PHA	85 339 ± 10 907	58 423 ± 7 074 (68 %)*
2	PHA	127 909 ± 8 002	111 244 ± 7 793 (87 %)
3	PHA	123 073 ± 11 802	88 061 ± 8 137 (72 %)
4	PHA	92 567 ± 13 103	77 287 ± 9 082 (83 %)
5	Fresh F cells	49 887 ± 7 231	40 907 ± 5 907 (82 %)
	Frozen F cells	39 855 ± 10 867 (80 %)	n. d.
6	Fresh F cells	77 517 ± 5 291	68 732 ± 12 605 (87 %)
	Frozen F cells	56 171 ± 6 835 (70 %)	50 077 ± 5 462 (65 %)
7	Fresh F cells	79 183 ± 7 751	59 076 ± 12 399 (75 %)
	Frozen F cells	57 163 ± 8 326 (72 %)	51 118 ± 7 742 (64 %)
8	Fresh F cells	30 656 ± 6 499	24 698 ± 5 474 (82 %)
	Frozen F cells	27 785 ± 4 861 (78 %)	21 459 ± 4 408 (70 %)

*) Parentheses indicates percentage of corresponding cultures with fresh cells only.

Acta path. microbiol scand. Sect. C 85 398–400 1977

FREEZING OF RAT LYMPHOCYTES

IV In vitro and in vivo responses of T-cells

Einar Hem

Laboratory for Haematology and Lymphology
Norsk Hydro s Institute for Cancer Research,
Montebello Oslo 3 Norway

Hem E. Freezing of rat lymphocytes. IV In vitro and in vivo responses of T-cells. Acta path. microbiol. scand. Sect C 85 498–400 1977

Rat lymph node lymphocytes have been frozen-thawed with 10 per cent dimethyl sulphoxide as cryoprotectant. The present study shows that these cells respond well in T-cell functional assays, such as stimulation with phytohaemagglutinin, as responder cells in the mixed lymphocyte reaction and in the local graft versus-host assay The magnitude of the responses obtained with frozen-thawed cells was some 70–80 per cent of corresponding fresh cells.

Key words Rat lymphocytes freezing T cells.

E Hem Laboratory for Haematology and Lymphology
Norsk Hydro s Institute for Cancer Research, The Norwegian Radium Hospital,
Montebello Oslo 3 Norway

Received 19 iv 77 Accepted 1 vi.77

In 1964 *Ashwood-Smith* (1) reported the successful preservation of viable mouse lymphocytes after slow freezing and rapid thawing in the presence of dimethyl sulphoxide (DMSO) This method has been shown to be feasible for freeze-thawing of both human and rodent lymphocytes. While information on T-cell function in frozen thawed lymphocytes, with respect to mitogen stimulation and mixed lymphocyte reaction (MLR) is considerable as regards mice and man (6 7 9 10) information from rats is lacking An earlier report from our laboratory has shown that rat B-cells, as tested by immunoglobulin producing plaque-forming cells and their progenitors, survive freeze-thawing in high numbers (5) The present study indicates that frozen thawed rat lymph node lymphocytes also respond well in T-cell functional assays.

Materials and Methods

Inbred, locally-grown Hooded rats and F hybrids from Hooded × AGUS (from Chester Beatty Cancer Institute) were used at an age of 9–12 weeks. Single cell suspensions were prepared from cervical, brachial and mesenteral lymph nodes in medium RPMI 1640 with Hepes buffer (Gibco-Biocult Glasgow Scotland) and washed three times. This medium was used for all handling of th cells, whereas cell cultures were performed in RPMI 1640 with bicarbonate buffer

The cells were suspended in RPMI 1640 with 20 per cent fresh rat serum (FRS) in 2.5 ml samples at a concentration of 10^8 cells/ml. Another 2.5 ml medium with 20 per cent dimethyl sulphoxide (DMSO) was added gradually during 2.5 min and the suspension was injected into teflon/kapton bags and allowed to equilibrate for a further 7.5 min at room temperature The bag was pressed between aluminium plates, and cooling took place in a Revco ultra-low freezer operating at −90 C. The bag was placed above the polystyrene for 10 min then in the bottom of the freezer for 10 min and finally stored in liquid nitrogen for 10 min. This gave a reproducible freezing rate of 2 /min to 0 C and 6 /min to −60 C when the bag was transferred to −196 C. Thawing was carried out in a waterbath at 37 C, and 45 ml RPMI 1640 with 10 per cent FRS was

Acta path. microbiol. scand. Sect. C, 85 401–405 1977

HUMORAL AND CELL-MEDIATED IMMUNITY TO HEPATITIS B VIRUS ANTIGEN IN A HAEMODIALYSIS-RENAL TRANSPLANTATION UNIT

PETER SKINHØJ, JAN ALDERSHVILE and FINN HARDT

Department of Clinical Chemistry, Bispebjerg Hospital,
Department of Nephrology, Rigshospitalet, and
Medical Department II, Kommunehospitalet, Copenhagen, Denmark

Skinhøj, P., Aldershvile, J. & Hardt, F. Humoral and cell-mediated immunity to hepatitis B virus antigen in a haemodialysis-renal transplantation unit. Acta path. microbiol. scand. Sect. C, 85 401–405 1977.

Hepatitis B surface antigen (HB_sAg), the corresponding antibody (anti-HB_s), the antibody to hepatitis B core antigen (anti-HB_c), and the cellular immune response to purified HB_sAg (leucocyte migration inhibition test, LMIT HB_sAg) were determined in 19 staff members, 11 long-term haemodialysis patients, and 22 renal transplant patients in a haemodialysis-renal transplantation unit. Past or present infection, as expressed by the presence of circulating anti-HB_s or HB_sAg, was found in 30 cases (58 per cent) (11 staff members, six dialysis patients and 13 transplant patients). Anti-HB_c was found in all the ten HB_sAg positive cases—all patients—and in 13 out of 20 anti-HB_s positive cases. Four staff members and seven patients had positive LMIT HB_sAg; of these only one patient had HB_sAg, whereas seven cases had anti-HB_s. Neither the antibody determination nor the leucocyte migration inhibition assay disclosed any significant difference between patients and staff which could explain the different course of the hepatitis B virus infection in the two groups.

Key words: Humoral and cellular immunity; hepatitis B virus; dialysis; renal transplant patients.

Peter Skinhøj, Department of Infectious Diseases, Rigshospitalet,
DK 2100 Copenhagen Ø, Denmark

Received 13. 77. Accepted 13. 77.

Viral hepatitis type B may be diagnosed immunologically by detection of several virus specific antigens: Hepatitis B surface antigen (HB_sAg) (*Prince* 1968), hepatitis B core antigen (HB_cAg) (*Almeida et al.* 1971) and by the e antigen system (HB_eAg) (*Magnius & Espmark* 1972). Previous hepatitis B infection may be identified by demonstration of the corresponding antibodies anti-HB_s or anti-HB_c (*Barker et al.* 1973, *Hoofnagle et al.* 1973). In vitro test for detection of the cellular immunity to HB_sAg has been developed recently (*Frei et al.* 1973, *Howlett & McGuigan* 1975, *Pettigrew et al.* 1972, *Tong et al.* 1975) and such assays have been used to identify further cases of previous infection (*Pettigrew et al.* 1972, *Reed et al.* 1974).

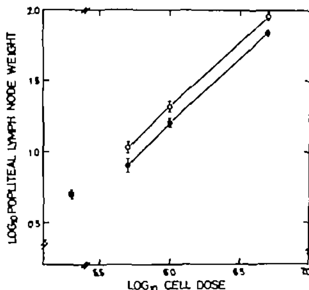


Fig 1 A local *graft versus-host assay with fresh* (–○–) or frozen thawed (–●–) Ho lymph node cells injected into foot pads of (Ho × AGUS) F_1 rats. ■ means of lymph node weight of normal controls. EEach point represents the log_{10} mean of 4–6 lymph nodes. Vertical bars represent ± 1 SD

stimulation are in agreement with those recently publiched by *Ortaldo et al.* (8) To the writer's knowledge, mixed lymphocyte reactions have not been reported when using frozen-thawed rat lymphocytes. Grant found that the effect of frozen-thawed *rat thoracic duct lymphocytes in the local* GVH assay was less than 30 per cent of that of fresh cells, compared to some 75 per cent in the present study (3)

The results obtained with frozen-thawed lymph node lymphocytes in these three different thymus dependent assays show that rat T-cell functions are well-preserved after freeze-thawing.

References 1 *Ashwood-Smith M J*. Blood 23 494–501 1964 —2 *Ford W L*, *Burr W & Simonsen M* Transplantation *10* 258–266, 1970. —3 *Grant C K* Clin. exp. Immunol. 23 166–174 1976.—4 *Hem E.* Cryobiology *13* 134–141 1976 —5 *Hem E* Acta path. microbiol. scand. Sect. C, *84* 489–494 1976 —6 *Jewett M A S Gupta, S Hansen J A Cunningham-Rundle S Siegal F P Good R. A. & Dupont B* Clin. exp. Immunol. *25* 449–454 1976.—7 *Oldham R. K., Dean J H., Cannon G B Ortaldo J R., Dunston G., Applebaum F McCoy J L., Djeu J & Herberman R B* Int. J Cancer *18* 145–155 1976 —8 *Ortaldo J R Oldham R. K Holden H T & Herberman R B.* Cell Immunol. *25* 60–73 1976.—9 *Strong D M., Woody J N Factor M A Ahmed A & Sell K W* Clin exp Immunol. *21* 442–455 1975 —10 *Thorpe P E Knight S C & Farrant J* Cryobiology *13* 126–135 1976 —11 *Yoshida, Y & Osmond D G* Transplantation *12* 121–129 1971

tents, as well as in 15 out of 20 anti-HB_s positive cases. No difference was found between patients and staff.

Leucocyte migration inhibition using purified HB_sAg was observed in ten cases only one of these was positive for HB_sAg. Seven of the cases had anti-HB_s simultaneously. Four cases of leucocyte migration inhibition were found among the 19 staff members, four among the 11 dialysis patients and two among 22 transplant recipients.

In the group of 20 anti-HB_s positive staff members and patients, LMT HB_sAg was apparently correlated with the time of infection. Among eight cases infected three months to two years previously four had leucocyte migration inhibition, whereas this was found in three out of 12 cases infected earlier (mean value of migration index 0.87 and 1.02, respectively).

Among the ten HB_sAg positive patients, only one had significantly reduced leucocyte migration. The migration index showed no correlation with the biochemical severity of infection in these patients.

DISCUSSION

The humoral immune response to an infection with hepatitis B virus comprises initially formation of antibodies directed against HB_cAg which develops very early after onset of the disease and persists with a high titre, i.e. detectable by complement fixation or electrophoretic tests in both persistent infection and for some time after recovery (*Hoofnagle et al.* 1973). Antibodies against HB_sAg raise more slowly but are detectable for many years, when sensitive haemagglutination or radioimmunological assays are employed (*Barker et al.* 1973, *Skinhøj & Steiness* 1975).

Determination of the cell-mediated immunity to hepatitis B virus (HBV) in man has been performed only by in vitro techniques, using HB_sAg as antigen. However a good correlation between in vivo and in vitro tests for HB_sAg has been found in experimental animal (*Ibrahim et al.* 1974). Variable results have been reported with the various tests employed: lymphocyte transformation (*Yeung Laiwah et al.* 1973), macrophage migration (*Houlett & McGuigan* 1975) and capillary tube or agarose leucocyte migration tests (*Frei et al.* 1973, *Ibrahim et al.* 1975, *de Moura et al.* 1975, *Tong et al.* 1975).

Lymphocyte stimulation or migration inhibition has been detected most consistently in the early convalescence stage after the infection (*Yeung Laiwah et al.* 1973, *Tong et al.* 1975, *Ibrahim et al.* 1975, *Aldershvile et al.*) but persistent reaction for long periods has been found (*Frei et al.* 1973, *Tong et al.* 1975). Application of these tests for evaluation of previous infection in high risk groups has been reported by two groups of investigators (*Lee et al.* 1975, *Pettigrew et al.* 1972, *Reed et al.* 1974). Both describe an extraordinarily high prevalence of cellular immunity to HB_sAg as compared to the occurrence of anti-HB_s, thus indicating a much wider dissemination of hepatitis B virus than estimated from tests for humoral immunity. However both in other recent studies (*Ibrahim et al.* 1975) and in the present study these findings could not be confirmed. LMT HB_sAg showed inhibition in 35 per cent of individuals positive for anti-HB_s and in three out of 22 without serological evidence of infection. This distribution is in accordance with the hypothesis that the cellular immune response to HB_sAg is most pronounced during the HB_sAg elimination phase of the infection (*Ibrahim et al.* 1975, *Aldershvile et al.*). Thus LMT HB_sAg is of little value as a marker for previous infection with HBV.

It has been postulated that the immune response to HBV antigens or in combination with liver membrane antigens causes the liver cell damage in the type B hepatitis. A decreased immune response should thus imply a subclinical course of the infection (*Edgington & Chisari* 1975).

In order to elucidate the reason for a different course of infection in patients as compared to staff members, the humoral and cellular immune responses to HBV were also

Whereas the occurrence of the viral antigens and antibodies during the infection are well established, the cellular immune response appears to be more variable, and the results published so far have been contradictory (*Ibrahim et al* 1975 *de Moura et al 1975 Tong et al* 1975)

The purpose of the present work was to evaluate both the humoral and the cellular immune response to hepatitis B virus antigens in staff members as well as in patients in a highly-exposed haemodialysis renal transplantation unit in order to elucidate a possible difference in the immune response which might explain the different course of the infection with hepatitis B virus in patients and healthy staff members.

PATIENTS AND METHODS

The dialysis unit studied, the dialysis regimen and the immunosuppressive treatment used in the renal transplant recipients have been described in previous reports (*Skinhøj* & *Steiness* 1975 *Steiness* & *Skinhøj* 1971) 19 regular staff members, 11 long term haemodialysis patients, and 22 renal transplant recipients were available for study Liver function tests, including determination of alanine-aminotransferase (GPT) bilirubin, alkaline phosphatases and prothrombin, as well as radioimmunological HB Ag determination, were performed at regular intervals on all patients and staff (*Skinhøj* & *Steiness* 1975)

Anti-HB was determined by a radioimmunoprecipitation assay described previously (*Skinhøj* & *Hansen* 1973) Antibody titre was estimated by the two-fold dilution technique

Anti-HB was detected by counter immunoelectrophoresis (*Skinhøj* & *Thulstrup* 1971) using purified HB Ag kindly provided by Dr *Bengt Göran Hansson* Malmö, Sweden. The electrophoresis was run for two hours in a 0.9 per cent agarose agar (5 1) gel, and the plates were read after overnight incubation without further treatment.

For detection of leucocyte migration inhibition, the one-step agarose technique of Clausen was employed (*Clausen* 1973)

Details of the assay modified for the present purpose will be given elsewhere (*Alderskile et al.*) The test antigen employed was the same purified HB_sAg preparation as that used for the radioimmunoprecipitation assay (*Skinhøj* & *Hansen* 1973) Leucocytes were preincubated for half-an-hour with HB_sAg at a concentration of 50 µg protein per ml the highest concentration showing no inhibition of leucocytes from healthy controls. The area of migration was read after 18 hours. In the present study significant inhibition of leucocyte migration was defined as a migration index less than 0 93 corresponding to the mean minus two standard deviations of 12 healthy controls (*Alderkile et al.*)

RESULTS

Table 1 shows the occurrence of anti-HB_c and LMT HB_sAg in relation to HB_sAg and anti HB_s in the three groups of subjects at the time of the investigation Overall infection rate, as indicated by presence of HB_sAg or anti HB_s was 30 out of 52 persons examined. However no case of antigenaemia was found among the staff Of the infected staff members, four out of 11 had had a clinically overt infection with jaundice, while this was the case in only four out of 19 patients.

No difference in the anti HB titres was found between the three groups (mean value 320 200 and 360 for staff dialysis patients and transplanted patients respectively Anti HB_s was present in all HB_sAg positive pa

TABLE 1 *Occurrence of HB_sAg and Anti-HB C related with Anti HB and LMT-HB_sAg in 33 Patients and 19 Staff Members*

	19 staff members			11 dialysis patients			22 transpl recipients		
HB category	HB Ag +	anti-HB +	0	HB_sAg +	anti HB +	0	HB_sAg +	anti-HB +	0
No. of cases	*0*	*11*	*8*	*3*	*3*	*5*	*7*	*6*	*9*
Anti-HB positive No. of cases	0	8	0	3	3	0	7	4	0
LMT HB Ag positive No. of cases	0	4	0	1	2	1	0	1	2

Cell-mediated immunoreactivity to hepatitis B surface antigen in liver diseases. Gastroenterology 69 310–317 1975.

Pettigrew N M Goodie R B, Russell R I & Chaudhuri A K R. Evidence for a role of hepatitis virus B in chronic alcoholic liver disease. Lancet 2 724–725 1972.

Prince A M An antigen detected in the blood during the incubation period of serum hepatitis. Proc. Nat. Acad. Sci. 60 814–821 1968

Reed W D Mitchell, C G Eddleston A L W F Lee W M Williams R & Zuckerman O J Exposure and immunity to hepatitis B virus in a liver unit. Lancet 1 581–583 1974

Singer D P S Rashid A M Laith, W A, Harris, J E, Couture R D & Sutherland M. Hepatitis B surface antigen (HB_sAg) infection in hemodialysis unit. II: Factors affecting host immune response to HB_sAg. CAM Journal 113 945–948 1975.

Skinhoj P & Hansen J F. Preparation and characterization of hepatitis B antigen for radioimmunoassay Scand J Clin. Lab. Invest. 32 199–203 1973

Skinhoj P & Steiness I Radioimmunoassay of hepatitis B antigen and antibody in dialysis and transplant recipients. Acta path. microbiol. scand Sect. B 83 125–128, 1975

Skinhoj P & Steiness I Epidemiologie de l'hepatite B dans une unité d'hemodialyse In "Hepatite a virus B et Hemodialyse" Flammarion, Paris 1975 pp. 143–148.

Skinhoj P & Thalstrup H. Hepatitis associated antigen. Scand. J Clin. Lab. Invest. 27: 61–68 1971

Steiness I & Skinhoj P. Hepatitis associated antigen. Elimination from a dialysis unit and persistence in renal transplant recipients. Acta path. microbiol. scand. Sect. B, 79: 721–725, 1971

Szmuness W Prince A M, Grady G F, Mann N K, Levine R W, Friedman E A, Jacobs, M J Josephson A, Ribot S Shapiro F L, Stenzel, K H, Suki, W N & Vyas G. Hepatitis B infection. JAMA 227 401–406 1974

Tong, M J, Wallace A M, Peters, R. L. & Reynolds T B. Lymphocyte stimulation in hepatitis B infection. New Engl. J Med. 283 318–322, 1975

Torisu M, Yokoyama, T, Amemiya H, Kohler P F Schroter G, Martineau G, Penn I, Palmer W, Malerison, C G, Putnam, C W & Starzl, T E. Immunosuppression, liver injury and hepatitis in renal, hepatic and cardiac homograft recipients. Ann. Surg. 1974 650–659 1971

Young Laiwah A A C, Chaudhuri A K R & Anderson J R. Lymfocyte transformation and leucocyte migration-inhibition by Australia antigen. Clin. exp. Immunol. 15 27–34 1973

compared within the three groups studied, namely 1) uraemic patients, 2) transplanted and immunosuppressed patients and 3) staff members. While in staff cases the infection is transient and usually clinically overt, subclinical but persistent infection occurs in 50 to 75 per cent of the patients (*London et al* 1969 *Torisu et al* 1971) The study failed to disclose any difference between the two groups as regards the anti HB$_s$ titres or the prevalence of anti HB$_s$, and although minor differences in antibody titres have been found in larger surveys (*Szmuness et al* 1974) the humoral responsiveness to HBV appears to be maintained in the dialysis and transplanted patients.

The prevalence of LMT HB$_s$Ag was low and did not disclose any significant difference in the cell mediated immunity between the groups. As contribution to the low prevalence of LMT HB$_s$Ag in the present series might also be the immunosuppressive treatment given to the transplant recipients (*Bendtzen* 1975) However with other antigens a normal cellular responsiveness has been found in dialysis patients and those treated with prednisone (*Clemmensen et al.* 1976 *Sengar et al* 1975) even the total number of T lymphocytes may be decreased in these patients (*Sengar et al* 1975)

In conclusion from the tests available for evaluation of immunity to HBV it was not possible to detect the immunological deterioration which causes persistent infection in the dialysis or transplanted patients.

The authors are greatly indebted to Dr *Ib Stenaes* and the staff at the Department of Nephrology Rigshospitalet, for their generous supply of clinical data and blood samples

Miss *Anne Marie Møller* and Miss *Alice Olsen* are thanked for excellent technical assistance

The study was supported by grants from *Ebba Celinders Foundation* and *Forskningsfonden for Storkøbenhavn Færøerne og Grønland*

REFERENCES

Aldershvile J Dietrichson O Hardt F Nielsen J O & Skinhøj P Humoral and cell mediated immunity to hepatitis B antigens in acute and chronic liver diseases Scand. J Gastroenterol. In press.

Almeida J D Rubinstein D & Scott E J New antigen-antibody system in Australia-antigen-positive hepatitis. Lancet *2* 1225-1227 *1971*

Bendtzen K Drug effect on human leucocyte migration and migration inhibitory activity from lymfocytes stimulated with Concanavalin A. Acta path. microbiol. scand. Sect. C, *83* 447-454 1975

Barker L F Peterson M R., Shulman N R. & Murray R Antibody responses in viral hepatitis type B. J Amer Med. Ass. *223* 1005-1008, 1973

Clausen J E Leucocyte migration agarose technique some technical details. Acta Allerg. *28* 351-364 1973

Clemmensen O Andersen V Hansen N E Karle H Koch C., Søborg M & Weeke B. Sequential studies of lymphocytes, neutrophils, and serum proteins during prednisone treatment. Acta. med. scand. *199* 105-111 1976.

Edgington T S & Chisari F V Immunological aspects of hepatitis B infection. Am. J Med. Sci. *270* 213-227 1975

Frei P C Erard P & Zinkernagel R. Cell-mediated immunity to hepatitis-associated antigen (HAA) demonstrated by leucocyte migration test during and after acute B hepatitis. Biomedicine *19* 329-383 1973

Hoofnagle J H Gerety R J & Barker L F Antibody to hepatitis-B virus case in man. Lancet *2* 869-873 1973

Howlett S A & McGuigan J E Inhibition of macrophage migration in response to hepatitis B antigen. Gastroenterology *69* 960-964 1975

Ibrahim A B. Vyas G N & Perkins H A Immune response to hepatitis B surface antigen. Infect. Immun. *11* 137-141 1975

Ibrahim A B Vyas G N & Prince A M Studies on delayed hypersensitivity to hepatitis B antigen in chimpanzees. Clin. exp Immunol. *17* 311-318, 1974

Lee W M Reed W D Mitchell C G Galbraith R M Eddelston A L W F., Zuckerman D J & Williams R Cellular and humoral immunity to hepatitis B surface antigen in active chronic hepatitis. Brit. med. J *1* 705-708 1975

London W T DiFiglia M Sutnik A & Blumberg B S An epidemic of hepatitis in a chronic-haemodialysis unit. New Engl. J Med. *281* 571-578, 1969

Magnius L O & Espmark J A New specificities in Australia antigen positive sera distinct from the Le Bouvier determinants. J Immunol *109* 1017-1021 1972

de Moura M C Vernace S J & Paronetto F

Cell-mediated immunoreactivity to hepatitis B surface antigen in liver diseases. Gastroenterology 69: 310-317 1975

Prigeon N M Caudle R. B Russell, R. I & Chandhari, A. K. R. E idence for a role of hepatitis virus B in chronic alcoholic liver disease Lancet 2 724-725 1972.

Prince, A M An antigen detected in the blood during the incubation period of serum hepatitis. Proc. Nat. Acad. Sci. 60 814-821 1968

Reed W D Mitchell C G Eddleston A L W F Lee W M Williams R & Z kerman O J Exposure and immunity to hepatitis B virus in liver unit. Lancet 1 581-583 1974

Sanger D P S Rashid A M Leith W A., Harris, J E., Couter R. D & Sutherland M Hepatitis B surface antigen (HB_sAg) infection in hemodialysis unit. II Factors affecting host immune response to HB_sAg. CAM Journal 113 945-948 1975

Skinhøj, P & Hansen J F. Preparation and characterization of hepatitis B antigen for radioimmunoassay Scand. J Clin. Lab. Invest. 32 199-203 1973

Skinhøj, P & Stirnass I Radioimmunoassay of hepatitis B antigen and antibody in dialysis and transplant recipients Acta path. microbiol. scand Sect. B, 83 125-128, 1975

Skinhøj, P & Stirnass I Epidemiologie de l'hepatite B dans une unité d'hemodialyse. I "Hepatite a virus B et Hemodialyse" Flammarion, Paris 1975 pp. 143-148.

Skinhøj P & Thelstrup H. Hepatitis associated antigen. Scand. J Clin. Lab. Invest. 27: 61-66, 1971

Stirnass I & Skinhøj P. Hepatitis associated antigen. Elimination from a dialysis unit and persistence in renal transplant recipients. Acta path. microbiol. scand. Sect. B, 79 721-725 1971

Szmuness W Prince A M Grady G F., Nan N K Levine R. W Friedman E. A., Jacobs M J., Josephson A., Ribot S Shapero F L., Stenzel, K. H., S H W V & Vyas, G. Hepatitis B infection. JAMA 227 401-406, 1974

Tong, M J Wallace A M., Peters, R. L. & Reynolds T B. Lymphocyte stimulation in hepatitis B infection. New Engl. J Med. 285 318-322, 1975

Torisu M., Yokoama T., Amemiya, H., Kohler P F., S kroter G., Martineau G., Penn I., Palms W., Malcrison C G Putnam C W & Sterzl, T E. Immunosuppression, liver injury and hepatitis in renal, hepatic and cardiac homograft recipients. Ann. Surg. 1974 630-639 1971

Yeu g Laiwah A. A C., Chaudhuri A. K. R. & Anderson J R. Lymfocyte transformation and leucocyte migration-inhibition by Australia antigen. Clin. exp. Immunol. 15 27-34 1973

Acta path. microbiol. scand Sect. C 85 406–412 1977

SURFACE MARKERS IN NON-PHAGOCYTIC HAIRY CELL LEUKEMIA

R. MATRE, I TALSTAD and A. HAUGEN

Broegelmann Research Laboratory for Microbiology
Medical Department B and Department of Pathology University of Bergen, Bergen, Norway

Matre, R. Talstad I & Haugen, A. Surface markers in non phagocytic hairy cell leukemia. Acta path. microbiol scand. Sect. C, 85 406–412 1977

By phase contrast and scanning electron microscopy hairy cells were demonstrated in the peripheral blood, bone marrow and spleen of a patient with hairy cell leukemia Immunofluorescent tests revealed IgD on the surface of 92 per cent, IgG on 76 per cent, IgM on 12 per cent and albumin on 95 per cent of the cells from the spleen. After overnight culture IgG and albumin were detected on 4 and 6 per cent of the cells respectively while the number of IgD and IgM positive cells persisted Fifty-two per cent of the hairy cells formed rosettes with erythrocytes sensitized with IgG antibodies (EA) whereas 70 per cent formed rosettes after trypsin and protease treatment The hairy cells did not form rosettes with erythrocytes sensitized with IgM antibodies and complement (EAC) or with sheep and mouse erythrocytes. Cryostat sections of spleen strongly adsorbed EA, whereas no adsorption occurred with EAC or sheep erythrocytes. The hairy cells did not phagocytize latex particles or ingest a strain of yellow staphylococci. The results suggest that hairy cells from this patient probably were of B-lymphocyte origin.

Key words Leukemia hairy cells surface markers.

R. Matre, Broegelmann Research Laboratory for Microbiology MFH bygget, N 5016 Haukeland sykehus, Bergen, Norway

Received 5 v 77 Accepted 24 77

Hairy cell leukemia or leukemic reticuloendotheliosis, is a chronic disease which clinically resembles chronic lymphocytic leukemia. However the susceptibility for infections is more marked lymph node enlargement less common and there is usually dry tap on bone marrow aspiration (3 5 8)

The origin and nature of the hairy cells have not been clearly defined. Demonstration of phagocytic capacity (2, 11) adhesive properties (8) and presence of certain enzymes (22) indicate a monocytic origin of the hairy cell On the other hand the detection of immunoglobulins on the hairy cells and the demonstration of *in vitro* synthesis of immunoglobulins strongly suggest that the cells are of B lymphocyte origin (8) Receptors for the Fc portion of IgG have been demonstrated on most hairy cells (15 16) whereas receptors for C3 have only been detected in a few cases (13) By the use of scanning electron microscopy (SEM) the hairy cells have been characterized as very similar to B lymphocytes (23) monocytes (12) or hybrid in nature with features of both lymphocytes and monocytes (21)

The present paper reports data obtained from a study of the hairy cells of a patient with hairy cell leukemia.

PATIENT AND METHODS

Case Report (J F 1039/76)

The patient, a 52 year old woman, got an acute febrile illness in January 1970, with antibiotic resistent pulmonary infiltrates. Serum samples revealed increasing titre of antibodies to *Toxoplasma gondii* as detected in indirect haemagglutination and dye test (27). The pulmonary infiltrates disappeared after 5 months of prednison treatment. Later the patient was anemic (Hb 80 g/l), leukopenic (0.6–1.2 10^9/l) with (0.5–0.8 10^9/l) mononuclear cells, and had recurrent pulmonary and urinary tract infections and an enlarging spleen. In January 1976 the patient felt weak. The spleen was palpable 7 cm below the costal margin. There was no lymph node or liver enlargement. Eighty per cent mononuclear leukocytes were found in the peripheral blood. Serum immunoglobulin concentrations (IgG, IgA, IgM) were within the normal range. There was dry tap on bone marrow aspiration. Bone marrow biopsy showed heavy infiltration of hairy cells which also was found by re-study of the bone marrow biopsy from 1970. Prednison therapy did not result in a sustained improvement and splenectomy was done. The spleen (1400 g) revealed diffuse infiltration of hairy cells, destroying the normal structure. The patient was able to leave the hospital 25 days after the operation in relatively good condition. However, the patient died 1 month later of septicemia.

At autopsy there was heavy infiltration of hairy cells in enlarged retroperitoneal lymph nodes and bone marrow and slight infiltration of hairy cells in the liver.

Isolation of Mononuclear Cells

Mononuclear cells were isolated from heparinized blood by centrifugation on a Ficoll-Isopaque gradient (Lymphoprep, Nyegaard & Co., Oslo) (4). Suspensions of spleen cells were obtained by gently squeezing spleen fragments into phosphate buffered saline pH 7.2 (PBS). The cells were washed three times in PBS before use.

In Vitro Culture

Mononuclear cells were suspended at 10 cells/ml in Eagle culture medium supplemented with 10 per cent foetal calf serum, L-glutamine and gentamycin (50 µg/ml) and incubated overnight at 37 C in humidified atmosphere of 95 per cent air and 5 per cent CO_2.

Enzymatic Treatment

Spleen cells (10 cells/ml in PBS) were incubated for 30 min at 37 C in the presence of 2 or 10 mg/ml trypsin (Type XI, Sigma Chemical Comp.) or 2 mg/ml protease (Type VI, Sigma Chemical Comp.). The cells were then washed three times in PBS and examined for EA-rosette formation.

Demonstration of Surface Marker

Membrane bound immunoglobulins were detected by direct immunofluorescence technique (20). The mononuclear cells were incubated at 4 C for 30 min with fluorescein-conjugated rabbit antisera specific for human IgG, IgM, IgD, C3 and albumin (DAKO-immunoglobulins a/s, Copenhagen, Denmark and Behringwerke, Marburg-Lahn, West Germany).

Fc receptor positive cells were enumerated by rosette assay with ox or sheep erythrocytes (E) sensitized with rabbit IgG antibodies (A) (EA) (14) and C3-receptor positive cells were enumerated with sheep erythrocytes (E) sensitized with rabbit IgM (A) antibodies and human complement (C) (EAC). The EAC indicator cells were prepared as described previously (19). EAC rosettes were detected by incubating 0.25 ml of lymphocyte suspension (2 10^6 cells/ml) with 0.25 ml EAC at 37 C for 30 min. The cells were gently resuspended and the percentage of rosette forming cells determined in counting chamber. IgMEA and E served as controls. T-lymphocytes were assessed by their ability to form rosettes with sheep erythrocytes (7, 17). For the demonstration of rosette formation with mouse erythrocytes, the technique of *Stathopoulos & Elliot* (25) was applied. In all rosette tests cells binding three or more indicator cells were considered as rosette forming cells.

Demonstration of E, F and Complement Receptors in Tissue Sections

The procedures with tissue sections was the same as described previously using the closed chamber technique (19, 28, 29).

Phagocytic Function Test

Phagocytosis of latex particles were examined according to the method described earlier (26). The ability of the hairy cells to ingest and kill a strain of yellow staphylococci was kindly studied by D. H. Ilum (24).

Scanning Electron Microscopy (SEM)

Cells from peripheral blood and splenic tissue were fixed for one hour in glutaraldehyde (2 per cent in 0.1 M cacodylate buffer pH 7.2), rinsed twice with cacodylate buffer, post-fixed in 1 per cent osmium tetroxide for ½ h, and subsequently rinsed twice in buffer. Rosettes were fixed in 1 per cent paraformaldehyde and 0.5 per cent glutaraldehyde in 0.1 M phosphate buffer pH 7.3 overnight at 4 C before post-fixation. The cells



Fig 1 Scanning electron micrograph of a hairy cell from the peripheral blood (× 11 000)

were collected on nuclepore membrane filter (pore size 2.0 μ). The samples were dehydrated in a graded series of ethanol for 10 min each and critical point dried according to *Anderson* (1). The specimens were vacuum-coated with gold palladium, and then examined with a PSEM 500 microscope at 25 kV. Micrographs were recorded on Kodak *Tri X-pan professional TXP 120 film.*

RESULTS

Mononuclear cell suspension from peripheral blood contained 10–15 per cent hairy cells, identified by phase contrast microscopy. The prominent and numerous cytoplasmic projections on the cells were also demonstrated by SEM (Fig 1). The majority of the cells seemed to have ruffled membranes, a number of ridge like projections and many microvilli on their surface. Thirty six per cent of the mononuclear cells formed rosettes with sheep erythrocytes, 29 per cent formed rosettes with EA and 18 per cent with EAC (Table 1).

TABLE 1 *Surface Markers on Mononuclear Cells from the Patient Compared to Controls*

	Per cent rosette-forming cells with		
	E	EA a)	EAC
Patient			
Peripheral blood (preoperative)	36	29	18
Spleen	11	52 (70)	0
Controls			
Peripheral blood b)	65	27	28
Spleen b)	31	37	43

a) = bovine erythrocytes sensitized with one agglutinating unit of rabbit antiserum.
b) = mean value detected in five normal subjects. Number in parenthesis = Percentage after trypsin or protease treatment of the cells.

Numerous hairy cells were also demonstrated by phase contrast and SEM in a tissue specimen from the spleen (Fig 2). Mono-

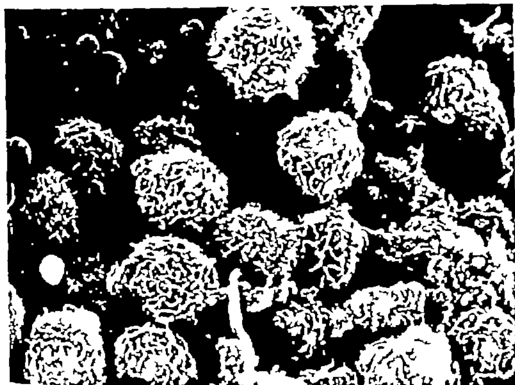


Fig 2 Scanning electron micrograph showing numerous hairy cells in a tissue specimen from the spleen (× 4000)

nuclear cell suspension from the spleen contained 85–90 per cent hairy cells. The SEM appearance of hairy cells from the spleen tissue was very similar to that from peripheral blood. Immunofluorescence revealed that IgD was present on 92 per cent and IgM on 12 per cent of the hairy cells before and after overnight culture. IgG was found on 76 per cent and albumin on 95 per cent of the hairy cells. However after overnight culture, IgG and albumin were detected only on 4 and 6 per cent, respectively.

The number of EA rosette forming cells increased from 52 to 70 per cent after trypsin or protease treatment. Enhanced rosette formation was also found when overnight cultured hairy cells were treated. SEM of an EA rosette forming hairy cell is shown in Fig 3. The hairy cells did not form rosettes with EAC, sheep (Table 1) or mouse erythrocytes.

Cryostat sections of the spleen showed strong adsorption of EA, covering the whole section. In sections from normal spleen the EA adsorbed to the red pulp and the periphery of the follicles. No adsorption occurred with EAC, or with sheep erythrocytes. Normally EAC shows strong adherence to the follicles and a weaker reaction with the red pulp, whereas E adhere around the central arterioles.

The hairy cells did not adhere to glass or plastic and they did not phagocytize latex particles or a strain of yellow staphylococci whereas monocytes from normal blood donors did.

DISCUSSION

The diagnosis of hairy cell leukemia in our patient was based on the clinical syndrome and the presence of mononuclear cells in the peripheral blood, spleen and bone marrow with a morphology similar to hairy cells.



Fig 3 Scanning electron micrograph of an EA rosette forming hairy cell (× 7000)

The presence of IgD and IgM on the cells before and after overnight culture strongly indicates a B lymphocyte origin of the hairy cell. IgG was most probably adsorbed onto the Fc receptors, since overnight culture effectively removed IgG (11 15). The presence of albumin on hairy cells has previously been demonstrated by *Huber et al* (15) and is very likely due to non specific sticking to the villous surface of the hairy cells

The Fc receptors on B lymphocytes are trypsin resistant but protease sensitive whereas the corresponding receptors on monocytes are resistant to both enzymes (10) Treatment of the hairy cells with these proteolytic enzymes enhanced the Fc receptor activity Consequently the Fc receptors on the hairy cells are similar to the corresponding receptors on the normal monocytes (18) However the cells from our patient did not have phagocytic or adhesive properties. This is inconsistent with a monocytic origin of the hairy cells. We believe therefore that our patient had a malignant proliferation of B lymphocytes. The hairy cells lacked receptors for sheep erythrocytes and activated C3 These data are in line with results reported by others (9 16) However *Haegert et al.* (13) did demonstrate C3 receptors on hairy cells in 3 of their patients.

Recently it has been found that rosette formation with mouse erythrocytes is a human B-cell marker (25) This marker however could not be detected on the hairy cells from our patient, which is in contrast to the results reported by *Catovsky et al* (6)

The results obtained when studying the morphology functional properties and immunological markers indicate that the hairy cells from various patients are heterogenous (Table 2) This heterogeneity may be explained by suggesting that in some patients

TABLE 2 *Immunological and Functional Properties of Hairy Cells*

Reference	N of cases	Cell suspensions							Sections of spleen	
		Surface immunoglobulins			Receptors for		Phagocytosis	Adherence	Receptors for	
		IgM	IgG	IgD	Fc	C3			Fc	C3
Debusscher et al.	1	+					—	+		
Dorgen et al	1			+	+	—			+	—
Fu et al.[11]	4	+	+)	+	+		+			
Hargert et al[13]	4	+	+	+	+	+	+			
Huber et al.[15]	4		+ b)		+	+	±			
Jaffe et al.[16]	2				+	—			+	—
Rosmasoja et al.[17]	3						+			
Present study	1	+	+)	+	+	—	—	—	+	—

Removed by overnight culture) or trypsin treatment b)
± = not carried out.

a proliferation of monocyte-like cells occur and in others proliferation of B lymphocyte-like cells occur. Alternatively hairy cell leukemia may involve proliferation of a subpopulation of B lymphocytes. The state of maturation of this cell would then determine the characteristics of the hairy cells. It is notable that Fc receptors have been detected on most hairy cells described so far (Table 2).

We are indebted to Dr *O T* der for helpful advice and discussion and to Dr *Hellum* for performing the phagocyte function test.
This work was supported in part by grant from the *Norwegian Cancer Society*.

REFERENCES

1. *Anderson T F*. Techniques for preservation of 3-dimensional structure in preparing specimens for electron microscopy. Trans N Y Acad Sci *13* 130–133 1951
2. *Barker C R, Burn G F, Cawley J C & Hayhoe F G J*. IgM receptors on the surface of hairy cells of leukaemic reticuloendotheliosis. Lancet *II* 1303–03 1976
3. *Burk J S, Byrn G E J & Rappaport H*. Hairy cell leukemia (leukemic reticuloendotheliosis) Cancer 33 1399 1410 1974
4. *Böyum, A*. Separation of leukocytes from blood and bone marrow. Scand J clin. Lab Invest. *21* Suppl 97 1968
5. *Catovsky D, Pettit J E, Galton D A G, Spiers A S D & Harrison C I*. Leukaemic reticuloendotheliosis ("Hairy cell leukaemia) A distinct clinico-pathological entity Brit. J Haemat. 26 9–27 1974
6. *Catovsky D., P pemichal, M., Okos A, Milians E & Holborow E. J*. Formation of mouse red cell rosettes by "hairy" cells. Biomedicine *23* 81–84 1975
7. *Coomb R R A, Gurner B. W, Wilson A. B., Holm, G & Lindgren B.:* Rosette-formation between human lymphocytes and sheep red cells not involving immunoglobulin receptors. Int. Arch. Allergy 39 658–663 1970
8. *Debusscher L., Bernheim J L., Collard-Ronge E., Govaerts A, Hooghe R., Lejeune F J, Zeicher M & Stryckmans P A*. Hairy cell leukemia. Functional immunologic, kinetic, and ultrastructural characterization. Blood *46* 495–506, 1975
9. *Dorgen, M J, Cossman, J, Chamey B T & Schecter B*. Hairy cell leukemia. An immunologic and ultrastructural study. Cancer *38* 1952–1961 1976.
10. *Dickler H B*. Studies of the human lymphocyte receptor for heat-aggregated or antigen-complexed immunoglobulin. J exp. Med. *140* 508–522, 1974
11. *F S M., Winchester R. J, Rai K. R & Kunkel, H G*. Hairy cell leukemia. Proliferation of cell with phagocytic and B-lymphocyte properties. Scand. J Immunol. *3* 847–851 1974
12. *Golomb H M, Braylan, R. & Pollack A*. "Hairy cell leukaemia (leukaemic reticuloendotheliosis) A scanning electron microscopic study of eight cases. Brit. J Haemat. 29 455–460, 1975
13. *Hargert D G, Cawley J C, Collin R. D., Flemans F J & Smith J L.:* Leukaemic re-

ticuloendotheliosis. A morphological and immunological study of four cases. J clin Path. *27* 967–972 1974

14 *Hallberg T Gurner B W & Coombs R. R A* Opsonic adherence of sensitized ox red cells to human lymphocytes as measured by rosette formation. Int. Arch. Allergy *44* 500–513 1973

15 *Huber C Asamer H Michlmayr G & Braunsteiner H* Surface immunoglobulins and receptor sites for aggregated IgG on leukemic reticuloendotheliosis cells Blut *32* 21–28 1976

16 *Jaffe E S Shevach E M Frank M M & Green I* Leukemic reticuloendotheliosis Presence of a receptor for cytophilic antibody Amer J Med *57* 108–114 1974

17 *Jondal M Holm G & Wigzell H* Surface markers on human T and B lymphocytes. I A large population of lymphocytes forming non immune rosettes with sheep red blood cells. J exp. Med. *136* 207–215 1972

18 *Lobuglio A & Rinehart J* In vitro and in vivo modification of human macrophage receptor for IgG globulin Clin Res. *18* 409 (Abstr) 1970

19 *Matre R & Tönder O* Complement receptors in human renal glomeruli. Scand. J Immunol *5* 437–441 1976

20 *Pernis B Forni L & Amante L* Immunoglobulin spots on the surface of rabbit lymphocytes J exp Med. *132* 1001–1018 1970

21 *Roath S & Newell D G* Cell-surface characteristics in hairy-cell leukaemia. Lancet *I* 283–284 1975

22 *Rosenszajn L A Gutman A, Radnay J, Ben David E & Shohem D* A study of the nature of "hairy" cells, with emphasis on enzymatic markers. Amer J clin. Path. *66* 432–441 1976.

23 *Schnitzer B & Hammack W J* B-lymphocyte nature of hairy cells in hairy-cell leukaemia. Lancet *ii* 649–649 1974

24 *Solberg, C O* Evaluation of neutrophil granulocyte functions. Acta path. microbiol. scand. Sect. B *80* 559–563 1972

25 *Stathopoulos G & Elliot E V* Formation of mouse or sheep red-blood-cell rosettes by lymphocytes from normal and leukaemic individuals. Lancet *i* 600–601 1974

26 *Talstad I* The relationship between phagocytosis of polystyrene latex particles by polymorphonuclear leucocytes (PML) and aggregation of PML. Scand. J Haemat. *9* 516–523 1972.

27 *Tönder O Closs O & Digranes A.* Comparison of the indirect haemagglutination and dye test for detection of antibodies to *Toxoplasma gondii*. Scand. J Infect. Dis. *6* 63–68, 1974

28 *Tönder O Milgrom F & Witebsky E* Mixed agglutination with tissue sections. J exp Med. *119* 265–274 1964

29 *Tönder O., Morse P A & Humphrey L J* Similarities of Fc receptors in human malignant tissue and normal lymphoid tissues. J Immunol. *113* 1162–1169 1974

Acta path. microbiol. scand. Sect. C, 85 413–423 1977

HUMAN LEUCOCYTE MIGRATION STUDIES WITH AN IMPROVED SKIN CHAMBER TECHNIQUE

KJELL B HELLUM and CLAUS O SOLBERG

The University of Bergen, School of Medicine, Medical Department B, Bergen, Norway

Hellum, K. B. & Solberg, C. O Human leucocyte migration studies with an improved skin chamber technique. Acta path. microbiol. scand. Sect. C, 85 413–423 1977

An improved skin chamber technique has been developed for the study of localized leucocyte mobilization (LLM) Uniform "windows" of denuded dermis were produced by a suction device applied to the forearm skin, eliciting delineated areas of epidermal separation by blister formation The acellular blister fluid, roof and basement membrane were removed, and the blister base was covered with a rubber chamber containing autologous serum as leucocyte attractant. Duplicate chambers were harvested at prescribed intervals during the first 24 hours. In 15 healthy individuals, virtually no cells were observed after 2 hours, median of 1.9×10^6 after 4 hours, increasing to 5.8×10 after 24 hours Subnormal LLM was demonstrated in three of seven patients with severe bacterial infections and in three of seven leukaemia patients. LLM was normal in eight patients with other malignancies. Ninety to 98 per cent of the cells were polymorphonuclear neutrophils and less than 1 per cent were erythrocytes In the chamber neutrophils, vacuolization of the cytoplasm was prominent, bactericidal capacity reduced and nitroblue tetrazolium reduction increased, thus indicating functional derangement of emigrated cells compared to peripheral blood neutrophils. Simplicity and good reproducibility should make this method valuable tool in the study of leucocyte migration.

Key words Human leucocyte migration in vivo suction blister; skin chamber granulocyte function.

K. B. Hellum, Medical Department B, 5016 Haukeland sykehus, Bergen, Norway

Received 23 77 Accepted 4 vii 77

Adequate migration of leucocytes to the sites of infection remains one of the corner stones of host defence, and infectious complications in acquired or inherited haematologic disorders have frequently been related to defects in leucocyte migration. Several of these defects have been diagnosed by *in vitro* studies of leucocyte chemotaxis, using Boyden type or "random" migration assays (13) However a major gap exists in the accurate clinical correlation of *in vitro* assays (13)

The study of localized leucocyte mobilization (LLM) *in vivo* using the "skin window" technique of *Rebuck & Crowley* (17) or the collection of peritoneal (20) tissue chamber (11) skin blister (2, 10) or chamber exudates (5 16 22) has the advantage of visualizing the cellular inflammatory response, but quantification and reproducibility may be

difficult due to complex experimental conditions. However using an improved skin chamber technique and high speed grinding for the production of skin lesions, *Senn* (18) has made a series of important studies of LLM. A skin chamber technique with epidermal abrasions produced by tape stripping also seems to be promising (12). However a major difficulty in using skin chambers is the production of standardized skin lesions which permit migration of sufficient numbers of leucocytes without undue tissue bleeding or breakdown. Recently *Kustala & Mustakallio* have drawn attention to the virtually non traumatic dermo-epidermal separation by suction blister production (9) thus providing regular areas of denuded dermis.

In the present study we have modified and combined the suction blister and skin chamber techniques, and developed an improved *in vivo* method for the measurement of leucocyte migration across intact tissue barriers.

MATERIALS AND METHODS

Subjects

The study comprised 22 patients (12 males and 10 females aged 16 to 76 years, median age 51 years) admitted to a medical department for bacterial infections or various malignancies (Table 1) and 15 healthy volunteers (11 males and 4 females aged 19 to 54 years, median age 34 years) among the hospital staff and medical students. Prior to investigation, penicillin G had been given for less than one day to two of the patients with bacterial infections. During the experimental period, antibiotic treatment was started (gentamicin 80 mg×3 and/or penicillin G 2 mill×4) in all but one of the infected patients. The patients with malignant diseases received neither chemotherapy nor radiotherapy prior to or during the study and none were being given corticosteroids or non-steroidal antiinflammatory compounds.

TABLE 1 *Diagnosis in 22 Patients with Acute Bacterial Infections or Various Malignancies Subject to Localized Leucocyte Mobilization Studies*

Diagnosis	Number of patients
Septicaemia	4
Lower respiratory tract infection	2
Cellulitis	1
Acute myeloblastic leukaemia	3
Acute lymphoblastic leukaemia	1
Chronic lymphocytic leukaemia	3
Lymphosarcoma	3
Hodgkin's disease	2
Liver carcinoma	2
Multiple myeloma	1



Fig 1 Polished perspex suction blocks with bores (A) outlet pipes (B) connection tubings (C) and sound thermistor (D)

Blister Suction Device

The suction device was composed of three parts 1) a suction pump (Egnell Universal, Pump AB Einar Egnell, Sweden) 2) plastic connection tubings arranged as a manifold with a series of Y-tube connectors, 3) suction blocks (Fig 1) with eight cylindrical bores and outlet pipes and a sound thermistor (type 2K2 Fenwal Electronics Inc., USA) connected to a precision ohm-meter (Digital multimeter model 134 Data Precision Corp., USA). The suction block, measuring 11.5 cm × 6.5 cm × 1.3 cm, was made of transparent plexiglass with polished bores 8 mm in diameter and rounded edges, each bore concentrically encompassed by a shallow groove at the bottom (skin) surface of the block. A short outlet pipe that would fit the tubings was inserted halfway down each bore and glued in place to make an air-tight connection. The thermistor was inserted to the centre of the skin surface of the block through a small extra bore and cemented with its tip just protruding through the surface to ensure good skin contact. Calibration of the thermistor and ohm meter between 30 C and 40 C was made by immersion of the suction block in a water bath.

Skin Blisters

The volar surface of each forearm was shaved and rinsed with 70 per cent isopropanol. A suction block was placed on the mid volar area and kept

in place by two rubber tourniquets. Since the dermo-epidermal adherence is highly influenced by skin temperature and suction pressure(8) a skin temperature of 37.0° C was maintained by 100 W heating lamp placed at a suitable distance from each block, and the suction pressure held at —0.3 kg/cm² throughout the blistering period. The blistering process was followed visually through the transparent suction block. Usually this started after about 90 minutes as minute blisters that gradually coalesced to form half spherical bulla as the dermo-epidermal separation reached the circumference of the bore. After 2 hours, when blistering was complete the suction device was detached. The clear slightly yellowish blister fluid was aspirated for cell counting. Using a sterile forceps, the epidermal blister roof and the glistening basement membrane were torn off at the blister circumference, leaving 0.5 cm² "skin window" as circular, sharply delineated areas of denuded dermis (Fig 2a). Usually no bruising was evident. However, in six subjects, minute intradermal haemorrhage occurred in the proximal window as evidenced by 3–8 scattered petechiae.

Skin Chambers

Sterile, tail-cutted hollow rubber stoppers (Chlorbutyl rubber Rubberfabriek Helvoet, N.V. Holland) with 16 mm internal diameter and cut-off flanges were used as chambers (Fig 2b). They could hold 2.0 ml and could be pierced with fine gauge needles without leakage. With the hollow side facing the skin, the stoppers were placed concentrically over the skin windows, glued (Cyanoacrylate Adhesive ACE E..., Alpha Techno Co. Japan) to the intact skin and firmly taped in position to avoid leakage.

Filling and Collection of Chamber Fluid

As attractant medium, 1.0 ml of fresh, autologous serum diluted 1:1 in Hanks' balanced salt solution (HBSS) was injected into each chamber through 23 gauge needle with another needle serving as a vent. The forearms were wrapped in elastic bandages to allow free use of the arms during the experimental period. Duplicate chamber exudates were harvested after 2, 4, 6, 8, 10, 22 and 24 hours by aspiration into plastic syringes containing 2.0 ml of HBSS. The chambers were flushed three times with the syringe content to wash out the cells. Complete emptying was confirmed by the presence of 3.0 ml of dilute chamber fluid in the syringe.

Occasionally when leakage was indicated by reduced fluid volumes, one or both spare chambers were harvested. With the exception of the 24-hour collection, emptied chambers were refilled with 1.0 ml of attractant medium stored at 4 C. To provide a surplus of fresh exudate cells for the

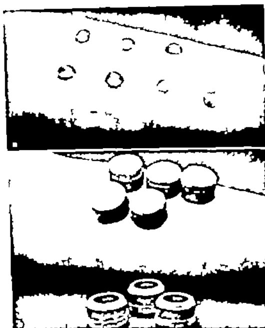


Fig 2a. Suction blisters and uncovered blister bases on the mid-volar area of the forearm.
b. Rubber skin chambers glued to intact skin surrounding the dermal blister bases.

functional tests (vide infra) all formerly harvested chambers were flushed and refilled at 22 hours and evacuated two hours later. At this time all chambers were removed, and the lesions were covered with vaseline gauze and dressings for three days.

Cell Count

Blister fluid and chamber exudate cells were counted, using an electronic particle counter (Celloscope 401, AB Lars Ljungberg & Co., Sweden) or a haemocytometer when the counts were less than 2×10^4 cells per ml. Counts are reported as the mean number of cells in duplicate chambers.

For differential counting, aliquots of all chamber fluids were spun in a cytocentrifuge (Cytospin, Shandon Southern Instruments Ltd., England) onto slides and treated with May-Grünwald/Giemsa stain. In each sample 100 cells were counted. Peripheral white blood cell (WBC) and differential counts were obtained from each subject at the start of the experiment.

Routine cultures of chamber fluid were made on blood agar plates incubated aerobically at 37 C for 24 hours.

Leucocyte Function Tests

At the end of the 24-hour chamber period ven ous blood was obtained from each patient and 11 of the healthy subjects, and was mixed with 10 units heparin per ml blood.

Viability

Viability studies on chamber exudate cells were performed by the trypan blue exclusion method (23)

Nitroblue Tetrazolium (NBT) Test

Histochemical NBT tests were made contemporaneously on peripheral blood leucocytes and on chamber leucocytes accumulated during the last 2 hour period of the chamber experiments. Unstimulated and endotoxin stimulated NBT tests were performed as described previously (6) Chamber exudate cells were spun down in plastic tubes for 5 minutes at 500 *g* and resuspended in 1 0 ml of heparinized autologous plasma (10 units heparin per ml plasma) before being subjected to identical test procedures as for heparinized whole blood.

Phagocytosis Test

The phagocytic and bactericidal capacity of peripheral blood and chamber leucocytes was studied contemporaneously The test procedure has been described in detail previously (7) Briefly a standard leucocyte-bacteria suspension containing 0.5 $\times 10^7$ neutrophil granulocytes, 2–3 bacteria (*Staphylococcus aureus*) per neutrophil and 10 per cent pooled human serum in HBSS was incubated at 37 C, and samples were removed periodically for determination of the total number of viable bacteria and the number of viable intracellular bacteria. The bactericidal capacity of the granulocytes is proportional to the total number of bacteria killed, and inversely proportional to the total number of viable bacteria or number of viable intracellular bacteria (21) The number of phagocytized bacteria equals the number of viable intracellular bacteria plus the number of bacteria killed (21)

Statistical Method

The Wilcoxon test for pair differences was used.

RESULTS

Blister Fluid

The blister fluid from both healthy subjects and patients was consistently acellular

Chamber Cell Counts

In the 15 normal subjects, virtually no cells were observed in the chamber fluid after 2 hours, 0.9–4 8 $\times 10^4$ (median 1.9 $\times 10^4$) after 4 hours, increasing to 22–80 $\times 10^6$ (median 38 $\times 10^6$) after 24 hours (Table 2 and Fig 3) The mean number of cells that migrated into each chamber per hour was 0 95 $\times 10^4$ during the 2–4 hour period 2.0 $\times 10^6$ during the 6–10 hour period and 1.5 $\times 10^6$ between 22 and 24 hours.

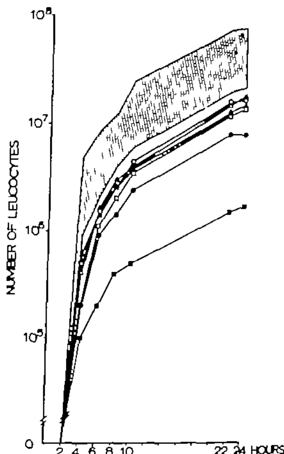


Fig 3 Leucocyte mobilization to skin chambers in three patients with septicaemia (open symbols) and three leukaemia patients (closed symbols) compared to normal leucocyte mobilization in 15 healthy individuals (hatched area) Each value represents the duplicate chamber mean.

In the patients with severe bacterial infections, the number of cells varied widely the median number per chamber mounting to 28×10^6 after 24 hours (Table 2) The maximum influx occurred between 8 and 10 hours, being 1 4 $\times 10^6$ cells per hour In three patients with septicaemia, cell mobilization was distinctly subnormal (Fig 3) In these patients the influx of cells between 6

TABLE 2 *Leucocyte Mobilization to Skin Chambers 15 H althy Individuals and 22 Patients with Bacterial I fections Leukaemia or other Malignancies*

Diagnosis	No. of subjects	Leucocytes per chamber × 10^4 median (range) Time of collection							Peripheral blood counts × 10 median (range)	
		2 hours	4 hours	6 hours	8 hours	10 hours	22 hours	24 hours	Total WBC	Polymorphonucl. neutrophils
Normal	15	0 (—0 002)	1.9 (0.9 4.8)	4 0 (2 1–8 6)	8.0 (4.0 12)	12 (6 0–25)	35 (20–75)	38 (22–80)	6.2 (5 4–8.1)	4 0 (3 1–5 9)
Acute bacterial infections	7	0 (—0 001)	1.3 (0.2 4 3)	3 1 (1 7–7.9)	3 2 (2 0 13)	8.0 (3 5–24)	26 (12 76)	28 (14–78)	11.5 (6.4–18.0)	9 6 (5 2–16.1)
Acute and chronic leukaemia	7	0 (—0 001)	1 2 (0.1–4 6)	3 2 (0 2–8.7)	6 1 (0.4–15)	8 5 (0 5–23)	25 (1.5–75)	27 (1 7 76)	17 4 (2.1 60 0)	3 9 (0.5–6 6)
Other malignancies	8	0 (—0.002)	2.0 (1 1–4 7)	4 1 (2.3–8 0)	8.5 (5 0–13)	12 (7.0–26)	36 (22 70)	37 (23–76)	7 4 (2.4 11.8)	4 6 (2.1–7.8)

Mean alues of duplicate chambers.
Area of epidermal lesion 0.5 cm and 1.0 ml of 50 per cent tologous serum in HBSS as chamber medium.

and 10 hours ranged from 0.5 to 0 8 $\times 10^6$ per hour All three patients had increased numbers of mature neutrophil granulocytes in the peripheral blood (8 1–13.5 $\times 10^3$ per mm^3)

In the leukaemia patients, the cell mobilization also varied widely (Table 2) and was reduced distinctly in three patients (Fig 3) Two of these patients had acute myeloblastic leukaemia and the third chronic lymphocytic leukaemia Total cell mobilization after 24 hours mounted to 1 7 $\times 10^6$ 8 0 $\times 10^6$ and 15 $\times 10^6$ respectively and the mobilization rate per hour ranged from 0 05–0 8 $\times 10^6$ in the 6 to 10 hour period However the number of mature granulocytes in the peripheral blood was low (0.5–2 1 $\times 10^3$ per mm^3)

In the remaining patients with malignancies cell mobilization was normal (Table 2)

The number of cells in each of duplicate chambers deviated from the mean by 1 to 40 per cent (mean 10 per cent)

Irrespective of collection time, the chamber cell population consisted of 90 to 98 per cent polymorphonuclear neutrophil granulocytes, including 5 to 10 per cent "band" forms 2 to 8 per cent monocytes and macrophages, and the eosinophils, lymphocytes or erythrocytes never exceeded 1 per cent Usually erythrocytes were not present Younger forms of the myeloid series were not observed, even in leukaemia patients with abundant immature circulating leucocytes.

Vacuolization of the cytoplasm was a prominent finding in chamber neutrophils from patients and healthy subjects. Necrobiotic leucocyte forms, characterized by pycnotic or droplet nuclei, represented less than 1 per cent at 4 and 6 hours, gradually increasing to 3–10 per cent (mean 8 per cent) after 24 hours. However in the fresh (? hour) exudates collected at the end of the chamber period less than 4 per cent necrobiotic neutrophils were seen.

Bacteriological Control

Routine chamber fluid cultures were all negative.

Viability Studies

92 to 98 per cent of the chamber leucocytes resisted staining with trypan blue. At 2 to 10 hours, only 2 to 4 per cent of the neutrophils were stained and the maximum of 8 per cent was demonstrated in 24-hour exudates.

NBT Tests

The tests were performed in the peripheral blood prom 11 healthy individuals and the percentage of NBT positive neutrophils in the unstimulated test ranged from 1 to 14 median 5 and in the endotoxin stimulated test from 32 to 76 per cent, median 43 per cent (Table 3) For chamber leucocytes, markedly elevated scores were demonstrated in the unstimulated test (25 to 62 per cent, median 38 per cent) The scores differed significantly from those in the peripheral blood ($P < 0.01$) Endotoxin stimulation of cham-

TABLE 3 *Unstimulated and Endotoxin Stimulated NBT Test in Peripheral Blood and Skin Chamber Neutrophils from 22 Patients with Bacterial Infections Leukaemia or other Malignancies and in 11 Healthy Subjects*

Diagnosis	No of subjects	Percentage of NBT positive neutrophils, median (range) Peripheral blood Unstimulated test	Peripheral blood Endotoxin stimulated test	Chamber exudate Unstimulated test	Chamber exudate Endotoxin stimulated test
Normal	11	5 (1–14)	43 (23–76)	38 (25–62)	48 (33–79)
Acute bacterial infections	7	32 (12–56)	61 (?5–91)	45 (30–70)	56 (38–81)
Acute and chronic leukaemia	7	7 (0–13)	38 (18–65)	37 (26–61)	50 (32–76)
Other malignancies	8	9 (2–24)	45 (21–82)	41 (24–68)	51 (26–80)

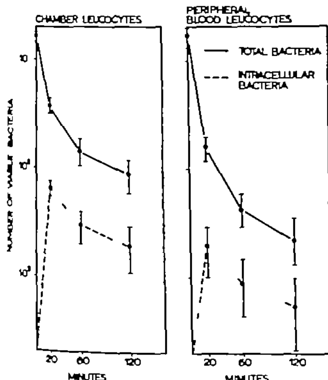


Fig 4 Number of viable bacteria during incubation with skin chamber and peripheral blood neutrophils from 11 healthy individuals (median and range)

ber leucocytes caused only moderately higher scores (range 33 to 79 per cent, median 48 per cent) These scores did not differ significantly from those in the peripheral blood (p > 0 1)

In the patients with bacterial infections and one patient with Hodgkin s disease, unstimulated NBT test scores in the peripheral blood were elevated, i.e more than 15 per cent NBT-positive neutrophils (6 7) In all patients, unstimulated NBT test scores in chamber exudates were increased to the same degree as those of the healthy subjects. The scores differed significantly from the test results in unstimulated peripheral blood (P < 0 01) Endotoxin stimulation of chamber leucocytes caused only minor increments in the number of NBT-positive cells (Table 3)

Phagocytosis Test

In the test with peripheral blood leucocytes from the healthy individuals, 0.5–2.3 per cent (median 1 4 per cent) of the bacteria remained viable after incubation for 120 minutes (Fig. 4) In contrast, 3.9–8.1 per cent (median 5 6 per cent) of bacteria in the test with chamber leucocytes remained viable after 120 minutes. At 20 60 and 120 minutes the values of the peripheral blood and chamber leucocytes differed significantly (P < 0.01) In the test with chamber leucocytes, large numbers of viable bacteria were located intracellularly (Fig. 4) thus demonstrating almost unimpaired phagocytosis but reduced intracellular killing of bacteria. Again, the values for the chamber and peripheral blood leucocytes were significantly different (P < 0.01)

Comparison of the phagocytic and bactericidal capacity of chamber and blood leucocytes in the patient group (Fig 5) showed similar differences as for the normal controls, i.e. significantly elevated numbers of total and intracellular viable bacteria in the tests with chamber leucocytes at 20 60 and 120 minutes (P < 0.01)

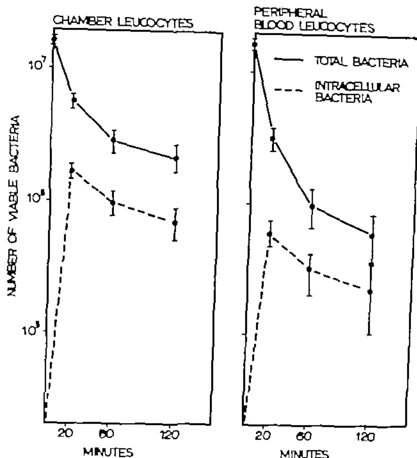


Fig 5 Number of viable bacteria during incubation with skin chamber and peripheral blood neutrophils from 22 patients with bacterial infections or malignant diseases (median and range)

Fate of Skin Lesions

Reepitelialization of the denuded blister base was completed within one week, leaving only faint dyspigmentation but no permanent scar

DISCUSSION

Production of skin blisters by local suction has been used mainly for dermatological investigations (3 8 9) but also lends itself to studies of the cellular dynamics of inflammation (10) The dermo-epidermal separation which occurs at the basement membrane (9) is a painless and aseptical procedure, and permits access to dermal windows when the blister roof and basement membrane are removed The 'windows are reproducible in terms of area and depth and signs of the dermal tissue and blood vessel damage which have hampered LLM studies using abrasive skin window techniques (18) have virtually not been demonstrated (9 10) In the present study blood vessel damage causing passive diapedesis of leucocytes also seems unlikely since erythrocytes were not demonstrated in the blister fluid or in the early chamber exudates. Moreover by avoiding tissue breakdown which may produce potent leucocyte attractants (19) interfering with the standard chamber medium, LLM should be influenced mainly by leucocyte and humoral factors.

Ordinary rubber stoppers for infusion bottles were chosen as chambers. They resist autoclaving and repeated filling and harvesting by needle puncture and do not introduce ports which might leak or allow contamination of the chamber fluid Furthermore, they are inexpensive and easily obtainable and their pliability prevents skin damage. Finally the 2 ml chamber volume permits the chamber medium (1 0 ml) to wash the skin "windows" during the chamber period, thus coun

teracting the formation of a membrane like cell accumulation at the dermal surface (18)

Autologous serum was chosen as attractant medium because it mimics physiological conditions and has previously been shown to be the most effective leucocyte mobilization factor (5 18) A 1 1 dilution in electrolyte solution has also been shown to give close to maximal LLM counts as compared to undiluted serum (18) and permits replacement of the electrolyte solution by various chemicals to be studied in future experiments.

Specific serum factors stimulating the in vivo leucocyte migration have been elucidated only partially and may include complement components or other heat labile factors (5 18, 24) as well as gamma globulin, albumin or other serum proteins (18, 19) Moreover subcellular leucocyte fractions or cell free chamber exudates have also been shown to enhance the in vivo LLM (5 18) In the present study accumulation of cellular factors in the chamber medium might explain the increase in leucocyte influx up to 8 or 10 hours The factors responsible for the apparent slowing down of leucocyte influx later on are doubtful Accumulated metabolic products, as evidenced by the increasing acidity of the medium, might possibly have inhibited leucocyte migration (4) or intoxicated the cells leading to increased cell death and lysis. However crude indicators of cell damage, i.e. increased trypan blue stained and necrobiotic cell forms, did not indicate any gross leucocyte damage or death

The present observations on the kinetics of LLM compare favourably with previous results of skin chamber studies (16, 18) We have confirmed a latency period of 2–4 hours, followed by a rapid increase in the number of emigrated leucocytes The median of 38×10^{6} leucocytes per 0 5 cm^2 window at 24 hours in healthy individuals is also comparable to the results of skin abrasion studies (18) and exceeds by far the number of cells obtained after skin stripping with adhesive tape (12) However we have not found the different patterns of migration kinetics in healthy individuals formerly suggested to be of clinical importance (18) Although a 24-hour collection of chamber leucocytes might be convenient, our patients with subnormal LLM counts differed clearly from the normal range at 4 and 6 hours, thus suggesting that the present LLM procedure may give early valuable information.

The chamber leucocytes consisted largely of neutrophil granulocytes (10 per cent or less were monocytes or other cell types) Accordingly the present chamber model deals predominantly with the acute phase of the inflammatory reaction, and provides a method for quantification of granulocyte migration, but not of other cell types. In contrast, the predominant mononuclear response called forth by the Rebuck skin "window" technique possibly represents a foreign body reaction.

Decreased LLM to skin chambers has been demonstrated previously in various malignancies, particularly leukaemia (16 18 19) in diabetes mellitus and advanced liver disease, in bacterial and viral infections, disseminated lupus erythematosus, pernicious anaemia, nocturnal haemoglobinuria and some immunodeficiencies (5 18, 19) In addition, a growing number of defects in the in vitro chemotaxis and random mobility have been reported (13) In the present study only three patients with bacterial infections had distinctly subnormal chamber leucocyte counts, and there was no correlation between peripheral WBC and chamber leucocyte counts either in the patients or in the controls, except for three leukaemia patients who had low numbers of circulating and chamber neutrophils. Also our patients did not receive drugs which are known inhibitors of leucocyte mobilization in vivo or in vitro (13 19) It remains to be proved whether the defective LLM was caused by intrinsic leucocyte dysfunction or by humoral inhibitors

Vacuolization of the cytoplasm was an outstanding feature of chamber neutrophil morphology Cytoplasmic granules, however were usually not prominent and certainly did not resemble toxic granules Corresponding morphological changes may be produced by in-

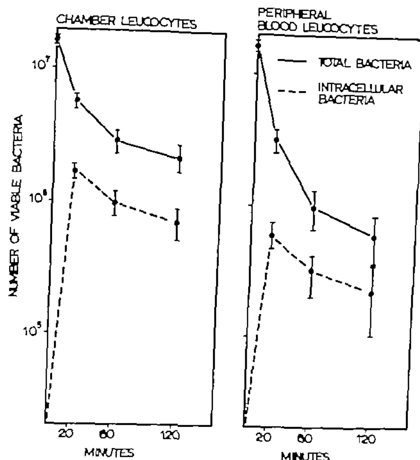


Fig 5 Number of viable bacteria during incubation with skin chamber and peripheral blood neutrophils from 22 patients with bacterial infections or malignant diseases (median and range)

Fate of Skin Lesions

Reepitelialization of the denuded blister base was completed within one week, leaving only faint dyspigmentation but no permanent scar

DISCUSSION

Production of skin blisters by local suction has been used mainly for dermatological investigations (3 8 9) but also lends itself to studies of the cellular dynamics of inflammation (10) The dermo-epidermal separation which occurs at the basement membrane (9) is a painless and aseptical procedure, and permits access to dermal "windows when the blister roof and basement membrane are removed. The "windows" are reproducible in terms of area and depth, and signs of the dermal tissue and blood vessel damage which have hampered LLM studies using abrasive skin window techniques (18) have virtually not been demonstrated (9 10) In the present study blood vessel damage causing passive diapedesis of leucocytes also seems unlikely since erythrocytes were not demonstrated in the blister fluid or in the early chamber exudates. Moreover by avoiding tissue breakdown which may produce potent leucocyte attractants (19) interfering with the standard chamber medium, LLM should be influenced mainly by leucocyte and humoral factors

Ordinary rubber stoppers for infusion bottles were chosen as chambers. They resist autoclaving and repeated filling and harvesting by needle puncture and do not introduce ports which might leak or allow contamination of the chamber fluid Furthermore they are inexpensive and easily obtainable and their pliability prevents skin damage. Finally the 2 ml chamber volume permits the chamber medium (1 0 ml) to wash the skin "windows" during the chamber period, thus coun-

events of blistering on human skin. J Invest. Derm. *48* 466-477 1967

10 *Kustala U Mustakallio K. K. & Rorsman H* Suction blisters in the study of cellular dynamics of inflammation. Acta derm.-venereol. (Stockh.) *47* 150-153, 1967

11. *Lindgren D & Lindhe J* Exudation, inflammatory cell migration and granulation tissue formation in preformed cavities. Scand. J plast. reconstr Surg. 7 1 9 1973

12 *Mass, M F Dean P B., Weston W L. & Humbert, J R.* Leukocyte migration in vivo a new method of study J Lab. clin. Med. *6* 1040-1046, 1975

13. *Miller M E.* Pathology of chemotaxis and random mobility Semin. Hematol. *12* 52-82 1975.

14 *Nathan D G Baehner R. L. & Weaver D K.* Failure of nitroblue tetrazolium reduction in the phagocytic vacuoles of leukocytes in chronic granulomatous disease J clin. Invest. *48* 1895-1904 1969

15. Park B H & *Good R A* NBT test stimulated Lancet *ii*. 616, 1970.

16 *Perillie P E. & Finch, S C* Quantitative studies of the local exudative cellular reaction in acute leukemia J clin. Invest. *43* 425-430 1964

17 *Rebuck, J W & Crowley J H* A method of studying leukocytic functions *in vivo* Ann N Y Acad. Sci (Wash.) *59* 757-805 1955

18. *Senn, H J* Infektabwehr bei Hämoblastosen: Funktionelle Untersuchungen über Leukocytenmobilisation beim gesunden und beim kranken Menschen. Exp Med. Path. Klinik Vol. *36* Springer Verlag, Berlin 1972

19 *Senn H J & Jungi W F.* Neutrophil migration in health and disease. Semin. Hematol. *12* 27-45 1975

20 *Snyderman R., Phillips J K. & Mergenhagen, S E.* Biological activity of complement in vivo. Role of C5 in the accumulation of polymorphonuclear leukocytes in inflammatory exudates. J exp. Med. *134* 1131-1143 1971

21 *Solberg C O.* Enhanced susceptibility to infection. A new method for the evaluation of neutrophil granulocyte functions. Acta path. microbiol. scand. Sect. B *80* 10-18 1972.

22. *Southam C M & Levine A. G.* A quantitative Rebuck technique. Blood *27* 734-738 1966.

23. *Tennant J R.* Evaluation of the trypan blue technique for determination of cell viability Transplantation *2* 685-694 1964

24 *Ward P A Cochrane C G & Müller Eberhard H J* Further studies on the chemotactic factor of complement and its formation *in vivo* Immunology *11* 141-153 1966

25. *Wintrobe M M* Clinical Hematology 7 ed. Lea & Febiger Philadelphia 1974 pp. 1278-1279

cubation of neutrophils *in vitro* and may represent phagosomes and autophagic vacuoles (25) Immature leucocytes were not demonstrated morphologically in the chamber exudates, even from patients with abundant leucocytes at various maturational stages in the peripheral blood. This observation supports the concept of participation in acute inflammatory reactions of mature leucocytes exclusively (18) However migrated myelocytes have been reported in chronic myelocytic leukaemia (19)

NBT dye reduction by phagocytes depends on the ingestive as well as the oxidative potential of the cells (14) the dye acting as a visible marker of phagocytosis and nucleotide mediated red-ox reactions (1) Increased formazan production by neutrophils after incubation with a standard endotoxin is used as an index of the functional capacity of these phagocytes (15) The results of unstimulated and endotoxin stimulated NBT tests on peripheral blood from our patients and controls were in accordance with previous studies, showing markedly elevated scores in patients with severe bacterial infections (6 7) In chamber neutrophils, however the unstimulated NBT test gave consistently elevated scores and endotoxin caused only minor enhancement of dye reduction. This indicates that these neutrophils were stimulated *in vivo* during migration or in the chamber possibly by serum or tissue factors, or by degradation products from the migrated leucocytes themselves. Also the phagocytosis tests showed alterations in the function of chamber as compared to peripheral blood neutrophils, i.e. significantly reduced intracellular killing of *S aureus* but only slightly impaired phagocytosis In a previous study of peripheral blod neutrophils from patients with severe bacterial infections, correlation between high NBT scores and reduced bactericidal capacity was demonstrated (7) This was caused possibly by increased *in vivo* phagocytosis resulting in enzymatic exhaustion of the interlocking killing mechanisms. The vacuolization of chamber leucocytes also lends support to this theory (7 25) The present results also suggest that migrating neutrophils become functionally worn out fairly rapidly and that an adequate host resistance depends on continuous emigration of fresh cells from the peripheral blood

The present *in vivo* skin chamber technique would seem to represent a physiologically relevant test for the study of leucotaxis, and defects could be further examined by more specific and elaborate *in vitro* migration techniques. Reproducibility and simplicity should enhance its use as a screening test for abnormal leucocyte migration.

The study was supported by grants from *The Norwegian Research Council for Science and Humanities* from *L. Meltzers Höyskolefond* and from *The Norwegian Cancer Society*

REFERENCES

1 *Baehner R L.* Use of the nitro blue tetrazolium test in clinical pediatrics. Amer J Dis. Child. *128* 449–451 1974
2. *Boggs D R., Athens J W., Haab O P Raab S O., Cartwright G E. & Wintrobe M M* Induced inflammatory exudates in normal man. A method designed to study the qualitative and quantitative cellular response to a pyogenic stimulus. Amer J Path *44* 61–71 1964
3 *Comaish S & McVittie E.* Suction blisters in bullous pemphigoid and other dermatoses. Brit. J Derm. *89* 127–132 1973
4 *Goetzl, E. J & Austen, F K* A neutrophil immobilizing factor derived from human leucocytes. I Generation and partial characterization. J exp. Med. *136* 1564–1580 1972.
5 *Gowland E* Studies on the emigration of polymorphonuclear leucocytes from skin lesions in man. J Path. Bact. *87* 347–352 1964
6 *Hellum K B. & Solberg C O* Influence of anticoagulants on the nitroblue tetrazolium test Scand. J Infect. Dis. *5* 67–70 1973
7 *Hellum K B & Solberg, C O* Granulocyte function in bacterial infections in man. Acta path. microbiol scand. Sect. C, *85* 1–9 1977
8. *Kiistala U* Dermal-epidermal separation II External factors in suction blister formation with special reference to the effect of temperature. Ann. clin Res. *4* 236–246, 1972.
9 *Kiistala U & Mustakallio K K* Dermo-epidermal separation with suction. Electron microscopic and histochemical study of initial

ing antibodies (*Robert et al.* 1973 *Jonker et al.* 1975, *Herva* & *Tiilikainen* 1977) where as antibodies reacting against relevant lymphocytes in the complement dependent cytotoxicity test are found in 10–20 per cent of such sera.

Some informative MLC reaction inhibiting multipara sera have been described in detail (*Gatti et al.* 1973, 1974 1975 *Revillard et al.* 1973) and possible mechanisms of MLC reaction inhibition by HLA-antisera have been discussed extensively in those papers (see also *Ceppellini* 1971 *Thorsby* 1974)

Three recent observations have given a firmer basis for this discussion (1) MLC stimulating HLA-D determinants are different from, although closely associated with, the serologically defined HLA-A, B, and C antigens, and unrelated HLA A, B, C identical subjects usually react against each other in MLC (see *Dupont et al.* 1976) (2) MLC reactions between HLA-A, B, C identical subjects can be inhibited by antisera against the stimulator cell *and* (3) these MLC stimulator inhibiting antisera are not cytotoxic for T-lymphocytes or non-fractionated lymphocytes in the conventional complement dependent lymphocytotoxicity test, but react with B-lymphocytes of the same stimulating cell donor in various test systems (*van Leeuwen et al.* 1973 see also *Wernet* 1976, *van Rood et al.* 1976)

This paper describes serum from a woman having had 17 children from 16 pregnancies and differing from her husband as regards only one HLA A and D specificity the other antigens being identical. This serum strongly and specifically inhibits MLC reactions, especially the stimulating function, and also responses to mitogen and antigen stimulation, but is not cytotoxic against non-fractionated mononuclear cells or enriched B-lymphocytes.

MATERIALS AND METHODS

Serum et, the Family of the Serum Producer and other Cell Donors

Serum *et* was found during studies concerning MLC reactions in pregnancy and the effect of maternal serum on these reactions (*Herva* & *Jouppila* 1977 *Herva* & *Tiilikainen* 1977) ET and her husband AT had 17 children, including a pair of dizygotic twins. ET was immunized by the pregnancies against the Rh determinants D and C, for which her husband AT is heterozygous. Beginning with the eighth child, seven of the infants had erythroblastosis and were treated by exchange blood transfusions. The highest anti-D and anti-C titres of serum *et* during the last pregnancy were 1 1024 and 1.32, respectively ET was in good general health, and there were no serious illnesses in the family

HLA antigens and MLC reactions of ET and AT were studied for the first time after the fifteenth pregnancy when the inhibitory effect of the serum was also found. Most of the results presented in this paper were from experiments performed after the birth of the twins (16th and 17th children) The serum lot in most experiments was drawn two and a half months after delivery This and other lots of the serum were prepared from clotted blood samples, stored in small aliquots at —20° C, and inactivated at 56 C for 30 minutes immediately before use in cultures diluted with at least an equal amount of fresh pooled male serum (*pms*)

In addition to ET and AT cells from their eighth (OT) ninth (NT) tenth (TT) thirteenth (KT) and fourteenth (MT) children were used in some experiments.

Other cell donors were members of our laboratory team and of three families from another study concerning HLA antigen associations in juvenile diabetics (*Åkerblom et al.* 1977) A homozygous HLA-Dw2 typing cell (*Ilonen et al.* 1977) was confirmed by Dr *Erik Thorsby* laboratory (Oslo Norway) and additional Dw2-homozygous cell were identified by typing reactions in both directions with this cell. Our tw provisionally identified HLA-Dw4-homozygous cells were from two families with diabetic children. HLA-Dw3 and Dw5 homozygous cells were identified by Dr *A ja Tiilikainen* (Helsinki, Finland)

Preparation of Mononuclear Cells and Enriched B-lymphocytes

Mononuclear cells were prepared from heparinized blood (*Bøyum* 1968 *Herva* 1977) B-lymphocytes were enriched by preparing sheep red blood cell rosettes as described from our laboratory (*Ryhänen* 1977) centrifuging the mixed cell suspension on a Ficoll-Isopaque discontinuous gradient (*Bøyum* 1968) and collecting the non-rosetted cells (approx. half lymphocytes, half monocytes) from the interface.

Complement Dependent Cytotoxicity Testing

The lymphocytotoxicity of serum *et* was tested by the two-stage microcytotoxicity test (*Amos et*

Acta path. microbiol. scand. Sect. C 85 424-434 1977

SPECIFICITY OF THE INHIBITORY EFFECT OF A GRAND-MULTIPARA SERUM ON MLC REACTIONS AND ON RESPONSES TO PHA, PWM AND PPD

ELJA HERVA and JORMA ILONEN

Public Health Laboratory Oulu, Finland

Herva, E. & Ilonen, J. Specificity of the inhibitory effect of a grand-multipara serum on MLC reactions and on responses to PHA, PWM and PPD. Acta path. microbiol. scand. Sect. C 85 424-434 1977

An MLC reaction inhibiting serum from a grand multiparous woman (16 successful pregnancies) is described. The serum producer had the HLA antigens A3,Bw35/Aw19,B18,Dw2 and her husband the antigens A28,Bw35/Aw19,B18,Dw2 and they responded to each other in one-way MLC. The serum had the following characteristics (1) it inhibited many but not all donors as stimulators in MLC (2) it usually inhibited these same donors as responders in MLC, but clearly to a lesser degree and with a much lower titre (3) the inhibition of the stimulating function followed HLA haplotypes in families, and seemed to have HLA D associated specificity inhibiting HLA Dw3 and Dw6 homozygous but not HLA-Dw2 or Dw4 homozygous cells as stimulators (4) it inhibited more strongly the PWM and PPD responses than the MLC responses of relevant cells (5) the IgG fraction of the serum had characteristics similar to the non-fractionated serum (6) the inhibitory effect on MLC reactions and PWM and PPD responses was not absorbable by specific red blood cells or platelets, but could be removed or diminished by specific lymphocytes (7) the serum was not cytotoxic in the complement-dependent test against non-fractionated mononuclear cells or enriched B-lymphocytes. The findings and the possible biological and clinical significance of human Ia-like antibodies are discussed.

Key words: Mixed lymphocyte reaction; mitogen response; specific inhibition; HLA-antisera; multiparity

Elja Herva, Public Health Laboratory, Kajaanintie 46 D, 90100 Oulu, Finland.

Received 4.v.77 Accepted 8.vii.77

Human mixed lymphocyte culture (MLC) reactions (for review see e.g. *Dupont et al.* 1976) can be inhibited by antibodies against HLA-determinants (*Leventhal et al.* 1970, *Ceppellini et al.* 1971, *Revillard et al.* 1972, 1973, *Gatti et al.* 1973, *Thorsby & Solheim* 1973). Antibodies against either the responding or stimulating cell can inhibit MLC reactions, but especially sera induced by multiparity inhibit more frequently and more strongly the stimulating function of the cells carrying the immunizing specificity (*Greenberg et al.* 1973, *Robert et al.* 1973).

About 50 per cent of the sera from multiparous women contain MLC reaction inhibit

ing antibodies (*Robert et al.* 1973 *Jonker et al.* 1975 *Hervа & Tiilikainen* 1977) whereas antibodies reacting against relevant lymphocytes in the complement dependent cytotoxicity test are found in 10–20 per cent of such sera.

Some informative MLC reaction inhibiting multipara sera have been described in detail (*Gatti et al.* 1973 1974 1975 *Revillard et al.* 1973) and possible mechanisms of MLC reaction inhibition by HLA-antisera have been discussed extensively in those papers (see also *Ceppellini* 1971 *Thorsby* 1974)

Three recent observations have given a firmer basis for this discussion (1) MLC stimulating HLA D determinants are different from, although closely associated with, the serologically defined HLA A, B, and C antigens, and unrelated HLA A, B C identical subjects usually react against each other in MLC (see *Dupont et al.* 1976) (2) MLC reactions between HLA A, B, C identical subjects can be inhibited by antisera against the stimulator cell *and* (3) these MLC stimulator inhibiting antisera are not cytotoxic for T-lymphocytes or non-fractionated lymphocytes in the conventional complement dependent lymphocytotoxicity test, but react with B-lymphocytes of the same stimulating cell donor in various test systems (*van Leeuwen et al.* 1973 see also *Wernet* 1976, *van Rood et al.* 1976)

This paper describes serum from a woman having had 17 children from 16 pregnancies and differing from her husband as regards only one HLA-A and D specificity the other antigens being identical. This serum strongly and specifically inhibits MLC reactions, especially the stimulating function, and also responses to mitogen and antigen stimulation, but is not cytotoxic against non-fractionated mononuclear cells or enriched B-lymphocytes.

MATERIALS AND METHODS

Serum et, the Family [of] the Serum Prod[uc]er and other Cell Donor[s]

Serum *et* w[as] found during studies [c]oncerning MLC reactions in pregnancy and the effect of maternal serum on these reactions (*Herv[a] & Jeppila* 1977 *Herva & Tiilikainen* 1977) ET and her husband AT had 17 children, including a pair of dizygotic twins. ET was immunized by the pregnancies against the Rh determinants D and C, for which her husband AT is heterozygous. Beginning with the eighth child, seven of the infants had erythroblastosis and were treated by exchange blood transfusions. The highest anti-D and anti-C titres of serum *et* during the last pregnancy were 1 10^4 and 1 32, respectively. ET was in good general health, and there were no serious illnesses in the family

HLA antigens and MLC reactions of ET and AT were studied for the first time after the fifteenth pregnancy when the inhibitory effect of the serum was also found. Most of the results presented in this paper were from experiments performed after the birth of the twins (16th and 17th children) The serum lot in most experiments was drawn two and [a] half months after delivery This and other lots of the serum were prepared from clotted blood samples, stored in small aliquots at −20° C, and inactivated at 56 C for 30 minutes immediately before use in cultures diluted with at least an equal amount of fresh pooled male serum (*pms*)

In addition to ET and AT cells from their eighth (OT) ninth (VT) tenth (IT) thirteenth (KT) and fourteenth (MT) children were used in some experiments

Other cell donors were members of our laboratory team and of three families from another study concerning HLA antigen associations in juvenile diabetics (*Akerblom et al.* 1977) A homozygous HLA-Dw2 typing cell (*Herva et al.* 1977) was confirmed by Dr *Erik Thorsby's* laboratory (Oslo Norway) and additional Dw2-homozygous cells were identified by typing reactions in both directions with this cell. Our two provisionally identified HLA-Dw4-homozygous cells were from two families with diabetic children. HLA-Dw3 and Dw6 homozygous cells were identified by Dr *Anja Tiilikainen* (Helsinki, Finland)

Pr[e]paration of Mononuclear Cells and Enriched B-lymphocyte[s]

Mononuclear cells were prepared from heparinized blood (*Boyum* 1968 *Herva* 1977). B-lymphocytes were enriched by preparing sheep red blood cell rosettes as described from our laboratory (*Rytkönen* 1977) centrifuging the mixed cell suspension on Ficoll-Isopaque discontinuous gradient (*Boyum* 1968) and collecting the non-rosetted cells (approx. half lymphocytes, half monocytes) from the interface.

Complement Dependent Cyto[to]xicity T[est]ing

The lymphocytotoxicity of serum *et* was tested by the two-stage microcytotoxicity test (*Amos et

al 1969) using rabbit complement. When B lymphocyte enriched suspensions were used as targets, incubations were lengthened from 15 min to one hour (serum *et* plus cells) and from 30 min to one hour (sensitized cells plus complement)

HLA Antigen Determinations

Serologically defined HLA antigens were typed by the microlymphocytotoxicity test (*Amos et al.* 1969) using the standard serum panel of the Finnish Red Cross Blood Transfusion Service obtained from Dr *Anja Tiilikainen*. HLA D typing was performed with homozygous cells(*Jørgensen et al.* 1973 *Ilonen et al.* 1977)

Lymphocyte Culture Techniques

Mixed lymphocyte culture (MLC) experiments were performed as reported previously (*Hervo* 1977) using if not otherwise stated microtitre plates with round-bottomed wells, each containing 0.50×10^5 responding and irradiated (6000 rad) stimulating cells per well The cultures were harvested on day five after labelling for 24 hours with ^{3}H-thymidine, and incorporated radioactivity was counted in a liquid scintillation counter Cultures (0.50×10^5 cells/well) stimulated with optimal concentrations of phytohaemagglutinin (PHA P Difco, 15 µg/ml) or pokeweed mitogen (PWM, GIBCO 12 µg/ml) were harvested on day three, and cultures with purified protein derivative of tuberculin (PPD Statens Seruminstitut, Copenhagen, 10 µg/ml) on day five as described from our laboratory (*Ryhänen* 1977) In all cultures, the medium (RPMI 1640 GIBCO) was supplemented with 20 or 25 per cent inactivated pooled male serum (*pms*) or dilutions of serum *et* in the same lot of *pms* The mean or median CPM values of triplicate cultures were used in calculating and presenting the results either as crude CPMs without subtracting background or as increment CPMs (test culture minus autologous control culture) Increment CPM values were used to calculate the inhibitory effect of serum *et* by the formula

$$\frac{\text{CPM in } pms \text{ minus CPM in the presence of serum } et}{\text{CPM in } pms} \times 100$$

Preincubation Experiments

Preincubation experiments were performed according to the principles of *Gatti et al.* (1974) 4×10^6 mononuclear cells were incubated in 0 5 ml of 1 4 dilution of serum *et* in *pms* or for control with 0.5 ml of *pms* for 45 min at 37 C and then for 45 min at 4 C, with shaking every 20 min. Thereafter the cells were washed three times at 4 C with medium supplemented with 5 per cent *pms* counted and cultured in suitable suspensions.

Absorptions

One millilitre of aliquots of serum *et* were absorbed with about 0.5 ml of a dense suspension of papainized red blood cells (known to contain Rh determinants D and C) with 10^9 platelets, or with 50×10^6 mononuclear cells, all from donor H, strongly inhibited as stimulator and clearly also as responder in MLC (see Results) Platelets (2×10^9/ml of serum *et*) from the husband (AT) were also used in one absorption experiment. Absorptions were performed at room temperature for one hour with frequent shaking 3 ml of *pms* was then added to each aliquot and the sera were centrifuged twice.

Preparation of IgG Fraction of Serum et

IgG was prepared (by *Hannu Suomela* Ph. D Helsinki, Finland) from serum *et* using a DEAE Sephadex A 50 ion exchange chromatography column and concentrated by ultrafiltration. The purity of the preparation was checked by immunoelectrophoresis, and its concentration was 47.2 g/l, as determined by radial immunodiffusion.

IgG was used in cultures diluted at least 1:4 with *pms* In this dilution the specific antibody concentration corresponded roughly to that of undiluted serum *et*

RESULTS

Effect of Serum et *on MLC Reactions between Serum Producer ET Her Husband AT and Unrelated Subjects*

In an MLC experiment performed six days after the birth of the fifteenth child cells ET and AT reacted with each other and with an unrelated control but all the reactions were strongly inhibited by serum *et* (lot 12/74) The inhibition was strongest against AT and unrelated H as stimulators but the responses of both donors against the serum producer were also clearly inhibited (Fig 1) The effect of serum *et* (10/76) on MLC reactions between ET AT and three unrelated donors (members of our group) eight days after the sixteenth delivery (twins) is shown in Fig 2 Responses of ET to AT H and P were strongly inhibited as were also other MLC combinations with AT H or P as stimulators. MLC combinations with ET or J as

stimulators were inhibited only moderately or weakly this inhibition was directed against the responders AT H and P in the case of ET and probably also of J J elicited only a weak typing response in ET and AT and the effect of serum *et* could not therefore be studied reliably in these combinations. The effect of serum *et* on the MLC reactions of H, P and J was confirmed in many experiments with these cells usually H was inhibited more strongly as responder than P

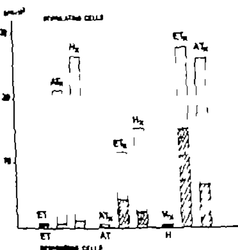


Fig. 1 MLC reactions between serum producer ET her husband AT and an unrelated donor H. 1.5×10^{5} responding and irradiated (5000 rad) stimulating cells per well were cultured on flat bottomed microtitre plates with 20 per cent pooled male AB-serum (whole columns) or with serum *et* diluted 1 2 with AB-serum (shaded parts of the columns) for five days. Results are presented as mean CPMs of triplicate cultures

Effect of Serum et *on MLC Responding and Stimulating Functions*

As described above serum *et* inhibited consistently and strongly H as stimulator and clearly also as responder at dilution 1:2 with *pms* On the other hand, J was inhibited neither as responder nor as stimulator Therefore MLC reactions between H and J were used to study the effect of various concentrations of serum *et* on the functions of H (Fig 3) Undiluted serum *et* (final dilution in cultures 1:4) strongly inhibited both functions, but especially the stimulating function. At dilution 1:4 with *pms* (final dilution 1 16) serum *et* inhibited the stimulating function

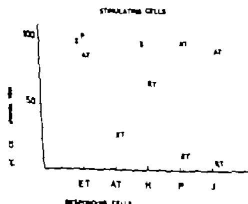


Fig 2 Effect of serum *et* on MLC reactions between serum producer (ET) her husband (AT) and three unrelated donors (H, P J) 0.5×10^{5} responding and irradiated (6000 rad) stimulating cells per well were cultured in round-bottomed microtitre plate wells for five days Percentage inhibition by serum *et* diluted 1 2 with pooled male serum (*pms*) of each MLC combination was calculated from median increment CPMs of *triplicate* cultures using the formula

$$\frac{\text{CPM in } pms \text{ minus CPM in } et}{\text{CPM in } pms} \times 100$$

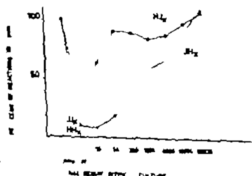


Fig. 3 Different effects of serum *et* dilutions on donor H as responder and stimulator in MLC. 0.5×10^{5} responding (H, J) and irradiated (6000 rad) stimulating (H_m J) cells per well were cultured in round-bottomed microtitre plate wells with 20 per cent pooled male serum (*pms*) or dilutions of serum *et* in *pms*. Results were calculated from mean CPMs of triplicate cultures and the reactions in the presence of serum *et* are presented as percentages of the reactions in plain *pms*

al 1969) using rabbit complement. When B lymphocyte enriched suspensions were used as targets, incubations were lengthened from 15 min to one hour (serum *et* plus cells) and from 30 min to one hour (sensitized cells plus complement)

HLA Antigen Determinations

Serologically defined HLA antigens were typed by the microlymphocytotoxicity test (*Amos et al.* 1969) using the standard serum panel of the Finnish Red Cross Blood Transfusion Service, obtained from Dr *Anja Tiilikainen*. HLA D typing was performed with homozygous cells(*Jørgensen et al.* 1973 *Ilonen et al.* 1977)

Lymphocyte Culture Techniques

Mixed lymphocyte culture (MLC) experiments were performed as reported previously (*Hervo* 1977) using if not otherwise stated, microtitre plates with round-bottomed wells, each containing 0.50×10^5 responding and irradiated (6000 rad) stimulating cells per well. The cultures were harvested on day five after labelling for 24 hours with ^3H thymidine and incorporated radioactivity was counted in a liquid scintillation counter. Cultures (0.50×10^5 cells/well) stimulated with optimal concentrations of phytohaemagglutinin (PHA P Difco 15 µg/ml) or pokeweed mitogen (PWM GIBCO 12 µg/ml) were harvested on day three, and cultures with purified protein derivative of tuberculin (PPD Statens Seruminstitut, Copenhagen, 10 µg/ml) on day five, as described from our laboratory (*Ryhänen* 1977). In all cultures, the medium (RPMI 1640 GIBCO) was supplemented with 20 or 25 per cent inactivated pooled male serum (*pms*) or dilutions of serum *et* in the same lot of *pms*. The mean or median CPM values of triplicate cultures were used in calculating and presenting the results either as crude CPMs without subtracting background or as increment CPMs (test culture minus autologous control culture). Increment CPM values were used to calculate the inhibitory effect of serum *et* by the formula

$$\frac{\text{CPM in } pms \text{ minus CPM in the presence of serum } et}{\text{CPM in } pms} \times 100$$

Preincubation Experiments

Preincubation experiments were performed according to the principles of *Gatti et al.* (1974). 4×10^6 mononuclear cells were incubated in 0.5 ml of 1:4 dilution of serum *et* in *pms* or for control with 0.5 ml of *pms* for 45 min at 37 C and then for 45 min at 4 C, with shaking every 20 min. Thereafter the cells were washed three times at 4 C with medium supplemented with 5 per cent *pms* counted and cultured in suitable suspensions.

Absorptions

One millilitre of aliquots of serum *et* were absorbed with about 0.5 ml of a dense suspension of papainized red blood cells (known to contain Rh determinants D and C) with 10^9 platelets, or with 50×10^6 mononuclear cells, all from donor H strongly inhibited as stimulator and clearly also as responder in MLC (see Results). Platelets (2×10^9/ml of serum *et*) from the husband (AT) were also used in one absorption experiment. Absorptions were performed at room temperature for one hour with frequent shaking. 3 ml of *pms* was then added to each aliquot and the sera were centrifuged twice.

Preparation of IgG Fraction of Serum et

IgG was prepared (by *Hannu Suomela* Ph.D Helsinki, Finland) from serum *et* using a DEAE Sephadex A 50 ion exchange chromatography column and concentrated by ultrafiltration. The purity of the preparation was checked by immunoelectrophoresis, and its concentration was 47.2 g/l, as determined by radial immunodiffusion.

IgG was used in cultures diluted at least 1:4 with *pms* in this dilution the specific antibody concentration corresponded roughly to that of undiluted serum *et*

RESULTS

Effect of Serum et *on MLC Reactions between Serum Producer ET Her Husband AT and Unrelated Subjects*

In an MLC experiment performed six days after the birth of the fifteenth child cells ET and AT reacted with each other and with an unrelated control, but all the reactions were strongly inhibited by serum *et* (lot 12/74). The inhibition was strongest against AT and unrelated H as stimulators but the responses of both donors against the serum producer were also clearly inhibited (Fig 1). The effect of serum *et* (10/76) on MLC reactions between ET AT and three unrelated donors (members of our group) eight days after the sixteenth delivery (twins) is shown in Fig 2. Responses of ET to AT H and P were strongly inhibited as were also other MLC combinations with AT H or P as stimulators. MLC combinations with ET or J as

stimulators were inhibited only moderately or weakly this inhibition was directed against the responders AT H and P in the case of ET and probably also of J J elicited only a weak typing response in ET and AT and the effect of serum *et* could not therefore be studied reliably in these combinations. The effect of serum *et* on the MLC reactions of H, P and J was confirmed in many experiments with these cells usually H was inhibited more strongly as responder than P

Effect of Serum et *on MLC Responding and Stimulating Functions*

As described above, serum *et* inhibited consistently and strongly H as stimulator and clearly also as responder at dilution 1:2 with *pms*. On the other hand, J was inhibited neither as responder nor as stimulator There fore, MLC reactions between H and J were used to study the effect of various concentrations of serum *et* on the functions of H (Fig. 3) Undiluted serum *et* (final dilution in cultures 1·4) strongly inhibited both functions, but especially the stimulating function. At dilution 1·4 with *pms* (final dilution 1 16) serum *et* inhibited the stimulating function

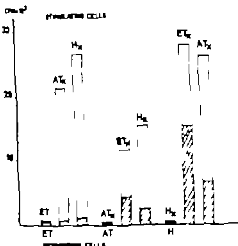


Fig 1 MLC reactions between serum producer ET her husband AT and an unrelated donor H. 1.5×10^5 responding and irradiated (3000 rad) stimulating cells per well were cultured on flat bottomed microtitre plates with 20 per cent pooled male AB-serum (whole columns) or with serum *et* diluted 1 2 with AB-serum (shaded parts of the columns) for five days. Results are presented as mean CPMs of triplicate cultures.

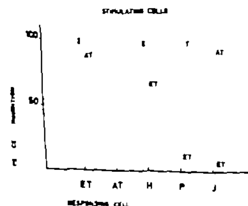


Fig 2 Effect of serum *et* on MLC reactions between serum producer (ET) her husband (AT) and three unrelated donors (H, P J) 0.5×10^5 responding and irradiated (6000 rad) stimulating cells per well were cultured in round-bottomed microtitre plate wells for five days Percentage inhibition by serum *et* diluted 1.2 with pooled male serum (*pms*) of each MLC combination was calculated from median increment CPMs of triplicate cultures using the formula

$$\frac{\text{CPM in } pms \text{ minus CPM in } et}{\text{CPM in } pms} \times 100$$

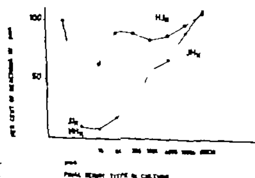


Fig. 3 Different effects of serum *et* dilutions on donor H as responder and stimulator in MLC. 0.5×10^5 responding (H, J) and irradiated (6000 rad) stimulating (H_x, J) cells per well were cultured in round-bottomed microtitre plate wells with 20 per cent pooled male serum (*pms*) or dilutions of serum *et* in *pms*. Results were calculated from mean CPM of triplicate cultures and the reactions in the presence of serum *et* are presented as percentages of the reactions in plain *pms*.

to the same extent but the responding function considerably less than undiluted *et*. Almost significant ($p < 0.05$) inhibition, calculated from the original triplicate CPMs by Student's t test occurred to a titre of 1:4096 (final dilution) on the stimulating function and to a titre of 1:16 (final dilution) on the responding function.

Preincubation of cells H in serum *et* with subsequent thorough washing clearly weakened their stimulating function but had no effect on their responding function. Similar treatment had no effect on the reactions of cells J (Table 1).

HLA haplotype and HLA D Specificity of the Stimulating Cell Inhibition by Serum et

In family T the paternal haplotype HLA A28,Bw35 (see Table 2) was the target for the stimulating cell inhibition by *et* (Table 3). This was also seen in another experiment (not shown) where ET responded to AT and their five children but the typing response of ET to three of the children (OT, NT and IT) made evaluation of the serum effect unreliable. ET and AT had HLA Dw2 in common, and the stimulating cell inhibition caused by serum *et* was directed against specificities of the other HLA haplotype of AT.

TABLE 1 *Effect of Preincubation of Responding and Stimulating Cells with Serum* et *on MLC Reactions*

Responding cell	Stimulating cell H_{pms}	H_t	J_{pms}	J_t	H_t	J_{mt}
H_{pm}	692	506	5388	5890		
H_t	362	409	4789	5304		
J_{pm}	9641	?872	336	200		
J_t	10916	4260	126	200		
H_t [b]					282	4206
J_t					10199	390

Lymphocytes E and J were preincubated at 37 C for 45 min at 4 C for 45 min with pooled male serum (pms) or serum *et* diluted 1:4 with pms, and washed three times at 4 C. Stimulating cells were irradiated with 6000 rad X rays, and cultures were incubated for 5 days.

Results are presented as median CPMs of triplicate cultures.

[b] H_t, J_t = *cells cultured simultaneously without pretreatment.*

TABLE 2 *HLA Antigens in Family T*

Father	AT	A28,Bw35/Aw19,B18,Dw2	a/b	(1)
Mother	ET	A3,Bw35 Aw19,B18,Dw2	c/b	
Child 8	OT	Aw19,B18,Dw2/Aw19 B18,Dw2	b/d	(2)
9	NT	Aw19,B18,Dw2/Aw19,B18,Dw2	b/d	(2)
10	IT	Aw19 B18,Dw /Aw19,B18,Dw2	b/d	(2)
13	KT	A28 Bw35/Aw19,B18,Dw2	a/d	(1)
14	MT	A28,Bw35/Aw19,B18,Dw2	a/d	(1)

(1) MLC reactions with each other absent or very weak.
(2) MLC reactions with each other absent or very weak; elicit typing response in other family members; react against maternal cells.

TABLE 3 *HLA-haplotype Specificity of the Stimulating Cell Inhibition by Serum et in Family T*

Responding cell donor	Stimulating cell donor: HLA-haplotypes		MLC reaction as increment CPM with pms[b]	with *et* 1:2	Inhibition %
P[d]	AT	a/b	7563	102	99
	OT	b/d	5120	4475	13
	NT	b/d	6750	4440	34
	IT	b/d	6295	4854	23
	KT	a/d	7494	183	98
	MT	a/d	5683	70	95

HLA antigens in Family T: see Table 2.

[b] pms = pooled male serum.

Serum *et* diluted with the same lot of pms.

[d] HLA antigens of P: A2,3,B8,w35,w6 (Dw3)

HLA-haplotype specificity of the stimulator inhibition by serum *et* was also seen in three families unrelated to family T. In the first family, the haplotype A9,B7,w6 was the target for the inhibition (Table 4). In the second family, the haplotype A10,Bw16 was inhibited, while in the third family the members carrying either of the maternal haplotypes A9,Bw40 or A2,Bw15,w6,Cw3 were strongly inhibited.

MLC responses of various donors to J and another Dw2-homozygous donor were inhibited only weakly, as were also the responses to two presumably Dw4-homozygous donors. On the other hand, responses to a Dw3-homozygous donor were inhibited strongly (by about 80 per cent) and to a Dw6-homozygous donor almost totally. These results are preliminary, but they show that the stimulating cell inhibiting effect of serum *et*, though specific for HLA-haplotypes, seems to be not restricted to any one HLA-D specificity as defined at present.

Effect of Serum et on the Responses to PHA, PWM and PPD

PHA responses of H, P and J were inhibited to about 60, 75 and 90 per cent, respectively, of control values by a final dilution of 1:16 of serum *et* (Fig. 4). PWM responses of H and P were also clearly inhibited (Fig. 4). The effect on the PWM response of P was somewhat variable: the values in the presence of serum et were from 17 to 76 per cent of control values. PPD responses of H and P were consistently and strongly inhibited by this concentration of serum *et*. The responses of J to PWM or PPD were not inhibited (Fig. 4).



Fig. 4. Effect of serum *et* on the responses of donors H, P and J to PHA, PWM and PPD. 0.5×10^6 cells per well cultured in round-bottomed microtitre plate wells for three (PHA and PWM) and five (PPD) days. Responses in the presence of 1:16 final dilution of serum *et* (shaded columns) and in plain *pms* (whole columns) are presented as mean CPMs of triplicate cultures.

to the same extent but the responding function considerably less than undiluted *et* Al most significant ($p < 0.05$) inhibition calculated from the original triplicate CPMs by Student's t test, occurred to a titre of 1 4096 (final dilution) on the stimulating function andto a titre of 1 16 (final dilution) on the responding function

Preincubation of cells H in serum *et* with subsequent thorough washing clearly weakened their stimulating function but had no effect on their responding function. Similar treatment had no effect on the reactions of cells J (Table 1)

HLA haplotype and HLA D Specificity of the Stimulating Cell Inhibition by Serum et

In family T the paternal haplotype HLA A28 Bw35 (see Table 2) was the target for the stimulating cell inhibition by *et* (Table 3) This was also seen in another experiment (not shown) where ET responded to AT and their five children, but the typing desponse of ET to three of the children (OT NT and IT) made evaluation of the serum effect unreliable. ET and AT had HLA Dw2 in common, and the stimulating cell inhibition caused by serum *et* was directed against specificities of the other HLA haplotype of AT

TABLE 1 *Effect of Preincubation of Responding and Stimulating Cells with Serum* et *on MLC Reactions*

Responding cell	Stimulating cell H_{pm}	H_{t}	J_{pms}	J_{t}	$H_{w t}$	J_{wt}
H_{pm}	692	506	5388	5890		
H_{t}	362	409	4789	5304		
J_{pm}	9641	2872	336	200		
J_{t}	10916	4260	126	200		
$H_{w t}$ [b]					282	4206
J_{t}					10199	390

Lymphocytes E and J were preincubated at 37 C for 45 min at 4 C for 45 min with pooled male serum (pms) or serum *et* diluted 1 4 with pms, and washed three times at 4 C. Stimulating cells were irradiated with 6000 rad X rays, and cultures were incubated for 5 days.

[a] Results are presented as median CPMs of triplicate cultures.

[b] $H_{w t}$ J_{t} = cells cultured simultaneously without pretreatment.

TABLE 2 *HLA Antigens in Family T*

Father	AT	A28,Bw35/Aw19,B18,Dw2	a/b	(1)
Mother	ET	A3,Bw35/Aw19,B18,Dw2	c/b	
Child 8	OT	Aw19,B18,Dw2/Aw19,B18,Dw2	b/d	(2)
9	NT	Aw19,B18,Dw2/Aw19 B18,Dw2	b/d	(2)
10	IT	Aw19,B18,Dw2/Aw19,B18,Dw2	b/d	(2)
13	KT	A28,Bw35/Aw19,B18,Dw2	a/d	(1)
14	MT	A28,Bw35/Aw19,B18,Dw2	a/d	(1)

(1) MLC reactions with each other absent or ery weak

(2) MLC reactions with each other absent or very weak elicit typing response in other family members react against maternal cells.

TABLE 5. *Effect of Abso ptions with Specific R d C lls Platelets or Lymphocytes on the I hibition by Serum et of Responses in MLC and to PHA PWM and PPD*

Responding cell	Stimulating agent	Median CPM of triplicate cultures in the presence of				
		pms	et$_u$	et$_{rc}$	et$_{pl}$	et$_l$
H	J	3776	3258	3579	3944	4382
	P	4839	1374	2030	1647	5256
	PHA	34720	21774	27165	29447	34211
	PWM	1504	724	780	448	1380
	PPD	2960	352	347	470	2304
P	J	6608	5943	3150	4361	7546
	H	6459	216	152	124	534
	PHA	34381	23823	28234	37160	51214
	PWM	5242	1166	750	944	3489
	PPD	7782	194	528	209	6354
J	P	9568	719	744	165	3660
	H_x	3202	322	784	821	949
	PHA	64279	34036	61707	58812	63138
	PWM	600	1633	1908	1328	1246

Cells from donors H P and J cultured with irradiated (6000 rad) stimulating (H_x,P_x,J_x) cells or PPD for fi days, or with PHA or PWM for three days in pms or absorbed serum *et*

pms = pooled male serum, et$_u$ = *et* unabsorbed, et$_{rc}$ = absorbed with red cells, et$_{pl}$ = with platelets, et$_{ly}$ = with lymphocytes All sera diluted 1 4 with pms.

For further explanations, see text

Revillard et al. 1973 *Brockner et al.* 1974) and especially the serum JH described by Gatti *et al* (1973, 1974 1975) being non-cytotoxic against (non-fractionated) lymphocytes of the specific immunizer Antisera produced by immunizing volunteers with serologically HLA identical but MLC non-identical cells seem to behave as the MLC reaction inhibiting multipara sera in MLC, having specificities against HLA D specificities or some determinants closely associated with these (*Albrechtsen et al.* 1977) Serum *et* is similar to serum TH of *Albrechtsen et al* (1977) in inhibiting the stimulating function of cells carrying HLA D specificities Dw3 and Dw6. Thus the specificity of this kind of MLC stimulator inhibiting sera seems to be broader than HLA-D specificities as defined at present, which may indicate possible cross-reacting groups among HLA-D determinants (see *Dupont et al* 1976)

In the mouse MLC stimulator inhibiting antisera are directed against Ia (immune response associated) antigens and react preferentially with B-lymphocytes (*Fish et al.* 1976, see also *Davies & Staines* 1976) In man, MLC stimulator inhibiting antisera react with the B-lymphocytes of specific donors in indirect immunofluorescence (*van Leeuwen et al* 1975 *Jonker et al.* 1975) in the complement dependent cytotoxicity test (*van Rood et al.* 1975) and in the Fc-receptor blocking test (see *Wernet* 1976) The human B-lymphocyte alloantigens detected by these methods are inherited in families together with HLA-D specificities, although they are somewhat broader. They have been called human Ia-like antigens (see *van Rood et al.* 1976) It is difficult, therefore, to understand the non-cytotoxicity of serum *et* against enriched B-lymphocytes of a donor strongly inhibited as MLC stimulator Whether this is due to non-sensitivity of our technique or to actual non-cytotoxicity of the serum remains

TABLE 4 *HLA-haplotype Specificity of the Stimulating Cell Inhibition by Serum* et *in Family N (Unrelated to Family T)*

Responding cell donor	Stimulating cell donor haplotypes		MLC reaction as CPM increment control serum	et serum 1·4	Inhibition %
J [b]	RN	a/b	1892	106	94
	MN	c/d	1580	4671	(stim. 196)
	PN	a/d	1548	70	95
	SN	a/d	1708	—0	100
	H		3050	124	96
H	RN	a/b	3990	184	95
	MN	c/d	2930	2685	8
	PN	a/d	3102	73	98
	SN	a/d	4244	138	97
	J		2782	2634	5

HLA haplotypes in family N a = A9,B7 w6 b = A3,B7 w6 c = A3 Bw35 w6 d = A2,B7 w6.

[b] HLA-antigens of J A1,2,B7 w6,Dw2

[c] HLA-antigens of H A2 w19,B27 w15 w4 w6

Effect of Absorptions with Red Blood Cells Platelets or Lymphocytes on the Inhibitions Caused by Serum et

Absorption of serum *et* with platelets from AT the husband of the serum producer did not weaken its inhibitory effect on the responses of ET to AT H or P (not shown) The serum *in this experiment was only dilut* ed 1 2 (final dilution 1 8) with *pms*

The effects of absorption of serum *et* with red blood cells platelets and lymphocytes (mononuclear cells) from H are shown in Table 5 *Absorptions with red blood cells or* platelets weakened only slightly and irregularly its effect on the reactions. Absorption with lymphocytes abolished the inhibitory effect on all reactions, except the MLC combinations with H as stimulator *It is noteworthy* that the effect on P as stimulator was either totally (in comb HP) or considerably (JP) *removed* by this absorption

Effect of IgG from Serum et *on lymphocyte responses*

Purified IgG fraction from serum *et* inhibited the MLC reactions and *PHA, PWM and* PPD responses of H, P and J as did the corresponding concentrations of the original serum *in terms of specificity* and degree of inhibition.

Complement Dependent Cytotoxicity of Serum et

The first sample of *et* (12/74) drawn about a week after the birth of the fifteenth child reacted weakly against AT mononu*clear cells in the complement dependent cy*totoxicity test This and a later lot (2/75) *also reacted weakly* and irregularly against cells having HLA A28 in common with AT (*Anja Tiilikainen* Helsinki Finland, personal *communication*)

The lots of serum *et* (10/76 and 12/76) drawn *after the birth of* the sixteenth and seventeenth children (twins) were not cytotoxic against mononuclear cells from AT H, P or J *The serum (12/76)* did *not* react against B-enriched lymphocytes from H and J in complement *dependent* cytotoxicity test.

DISCUSSION

In many respects serum *et* is similar to MLC *reaction* inhibiting pregnancy *induced sera* described by others (*Greenberg et al.* 1975

TABLE 5. *Effect of Absorptions with Specific Red Cells, Platelets or Lymphocytes on the Inhibition by Serum et of Responses in MLC and to PHA PWM and PPD*

Responding cell	Stimulating agent	Median CPM of triplicate cultures in the presence of				
		pms	et	et_{re}	et_{pl}	et_{ly}
H	J	3776	3258	3579	3944	4382
	P	4839	1374	2030	1647	5256
	PHA	34720	21774	27163	29447	34211
	PWM	1504	724	780	448	1380
	PPD	2960	352	547	470	2304
P	J	6608	5943	3150	4561	7546
	H_x	6459	216	152	124	534
	PHA	54581	25823	28234	37160	51214
	PWM	5242	1166	750	944	5489
	PPD	7782	194	528	209	6354
J	P	9568	719	744	165	3660
	H_x	3202	522	784	821	949
	PHA	64279	54036	61707	58812	63138
	PWM	600	1655	1908	1328	1246

Cells from donors H, P and J cultured with irradiated (6000 rad) stimulating (H_x $P_x$$J_x$) cells or PPD for five days, or with PHA or PWM for three days in pms or absorbed serum et.

pms = pooled male serum, et_u = et unabsorbed, et_{re} = absorbed with red cells, et_p = with platelets, et_{ly} = with lymphocytes All sera diluted 1 4 with pms

For further explanations, see text

Reillard *et al.* 1973 *Brockner et al* 1974) and especially the serum JH described by *Gatti et al.* (1973, 1974 1975) being non-cytotoxic against (non-fractionated) lymphocytes of the specific immunizer Antisera produced by immunizing volunteers with serologically HLA identical but MLC non-identical cells seem to behave as the MLC reaction inhibiting multipara sera in MLC, having specificities against HLA D specificities or some determinants closely associated with these (*Albrechtsen et al.* 1977 Serum et is similar to serum TH of *Albrechtsen et al* (1977) in inhibiting the stimulating function of cells carrying HLA-D specificities Dw3 and Dw6 Thus the specificity of this kind of MLC stimulator inhibiting sera seems to be broader than HLA D specificities as defined at present, which may indicate possible cross-reacting groups among HLA D determinants (see *Dupont et al* 1976)

In the mouse MLC stimulator inhibiting antisera are directed against Ia (immune response associated) antigens and react preferentially with B-lymphocytes (*Fish et al* 1976, see also *Davies* & *Staines* 1976) In man, MLC stimulator inhibiting antisera react with the B-lymphocytes of specific donors in indirect immunofluorescence (*van Leeuwen et al.* 1975 *Jonker et al.* 1975) in the complement dependent cytotoxicity test (*van Rood et al.* 1975) and in the Fc-receptor blocking test (see *Wernet* 1976) The human B-lymphocyte alloantigens detected by these methods are inherited in families together with HLA D specificities, although they are somewhat broader They have been called human Ia like antigens (see *van Rood et al* 1976) It is difficult, therefore, to understand the non-cytotoxicity of serum et against enriched B-lymphocytes of a donor strongly inhibited as MLC stimulator Whether this is due to non-sensitivity of our technique or to actual non-cytotoxicity of the serum remains

to be demonstrated In our preliminary experiments, serum *et* inhibited by about 50 per cent the Fc receptors of relevant lymphocytes (*Herva & Ryhänen* unpublished)

As far as we know the MLC stimulator inhibition test has not been compared systematically with other B-lymphocyte tests in sensitivity and specificity for the Ia like antibodies, although a clear correlation between MLC stimulator inhibition and indirect immunofluorescence tests has been found (*Jonker et al* 1975)

Two earlier lots of serum *et* (12/74 and 2/75) were weakly cytotoxic in the complement-dependent test against the mononuclear cells of AT the specific immunizer and also of some other donors carrying HLA A28 in common with AT Thus, the inhibitory effect of *et* on responding cells could be due to anti HLA A28 not detectable in the complement-dependent test but reacting in the more sensitive (*Bondevik et al* 1975) MLC reaction inhibition test On the other hand, sera produced by immunizing volunteers with HLA A B identical but HLA D non identical donors cells inhibited to some extent responder cells also (*Albrechtsen et al* 1977)

PPD (and PWM) responses of relevant cells were inhibited much more strongly than MLC responses of these cells by corresponding concentrations of serum *et* This confirms the observations of *Brochier et al* (1974) who suggested that the inhibition of PPD responses might be directed against Ia antigens on the responding lymphocytes.

Antibodies against human Ia like determinants are clinically important for at least three reasons firstly they could possibly be used in typing HLA D determinants serologically secondly they are similar in many respects to rodent Ia antibodies, which can induce prolonged graft survival (enhancement) (for reference and discussion see *Davies & Staines* 1976) and human Ia like antibodies have been suggested for clinical use (*Staines & Davies* 1975) thirdly in most HLA-disease associations B-lymphocyte (including HLA D) alloantigens are clearly more important than serologically defined HLA A, B C antigens (see Lancet 1976)

Multiple human pregnancies seem to be suitable stimuli for the production of MLC stimulation inhibiting antisera (*Robert et al.* 1973 *Jonker et al.* 1975 *Korithavongs et al* 1975 *Herva & Tuliikainen* 1977) Our observations on serum *et* suggest that extreme hypersensitization by pregnancies may lead to the disappearance of complement-dependent cytotoxicity even against B-lymphocyte antigens.

The authors wish to thank Mrs. *Kaarina Järvenpää* for technical assistance and Miss *Raili Liukko* for preparing the manuscript and drawings.

REFERENCES

Albrechtsen D Solheim B G & Thorsby E. Antiserum inhibition of the mixed lymphocyte culture (MLC) interaction. Inhibitory effect of antibodies reactive with HLA D-associated determinants. Cell. Immunol. *28* 258–273 1977

Amos D B, Bashir H Boyle W MacQueen M & Tiilikainen A A simple microcytotoxicity test. Transplantation *7* 220–222, 1969

Bondevik H., Albrechtsen D., Solheim B. G & Thorsby E Dissociated antibody inhibition of responding and stimulating cell in one-way mixed lymphocyte cultures (MLC) In *Kissmeyer Nielsen F* (Ed.) Histocompatibility testing Munksgaard, Copenhagen, 1975 p. 604–607

Brochier J Rotti I M & Festenstein H Inhibition of lymphocyte proliferative responses by anti-HL-A alloantisera. Eur J Immunol. *4* 709–715 1974

Boyum A Isolation of mononuclear cells and granulocytes from human blood. Scand J clin. Lab Invest. *21* suppl. 97 77–89 1968.

Ceppellini R Old and new facts and speculations about transplantation antigens of man. In *Amos B D* (Ed) Progress in Immunology Academic Press, New York and London, 1971 p 973–1025

Ceppellini R Bonnard G D Coppo F., Miggiano V C., Pospisil, M Curtoni E. S & Pellegrino M Mixed leukocyte cultures and HL A antigens. II Inhibition by anti-HL-A sera. Transplant. Proc *3* 63–70 1971

Davies D A L. & Staines N A A cardinal role for I region antigens (Ia) in immunological enhancement, and the clinical implications Transplant. Rev *30* 18–39 1976.

Dean J H Silva J S., McCoy J L. Leonard C M Cannon G B. & Herberman R B

Functional activities of rosette separated human peripheral blood leukocytes. J Immunol. *115* 1449–1455, 1975.

Dupont B Hansen J A. & Yunis E. J.: Human mixed-lymphocyte culture reaction Genetics, specificity and biological implications. Adv. Immunol. *23* 107–202, 1976.

Fuks, F Starzer, \ A Swern J L. & Davies D A. L.: The effects of erythrocyte-absorbed H-2 alloantisera on mixed lymphocyte culture reactions. Transplantation *22* 551–558, 1976

Gatti, R. A Yunis, E J & Good R. A Characterization of serum inhibitor of MLC reactions. Clin. exp. Immunol. *15* 427–437 1973.

Gatti, R A S edmy E. A J & W gzell H.: Characterization of serum inhibitor of MLC reactions. II Molecular structure and dissociation of inhibition against responder and stimulator function Cell Immunol *11* 466–477 1974

Gatti, *R A S dmy E A J Leipold W & Wigzell H* Characterization of a serum inhibitor of MLC reactions. III Specificity HL-A related antibodies Cell. Immunol. *15* 432–451 1975

Greenberg, L. J Rasmussen \ & Yunis E. J Dissociation of stimulation (MLR-S) and response (MLR-R) in mixed leukocyte culture by serum blocking factors. Transplantation *16* 520–522, 1973

Herv E. Comparison of microtitre plates with flat-bottomed and round-bottomed wells for mixed lymphocyte culture (MLC) Acta path. microbiol scand. Sect C, *85* 90–98, 1977

Herv E & Jouppila, P Mixed lymphocyte culture reactions between parental cells in pregnancy and puerperium. Acta path. microbiol. scand. Sect C, *85* 99–106, 1977

Herv E & Tukkanen, A Mixed lymphocyte culture reactions t delivery and in the puer perium Effects of parity HLA antigens and maternal serum Acta path. microbiol scand. Sect C, *85* 333 344 1977

Hämmerli g, G J Tissue distribution of Ia antigens and their expression on lymphocyte subpopulations Transplant. Rev *30* 64–82 1976

Ilonen J *Herv E Res ssen M Panelius M Meurman O A ula P & Tukkanen A* HLA antigens and antibody responses to measles and rubella viruses in multiple sclerosis Acta Neurol. Scand *55* 299–309 1977

Jonker M Persijn G G & an Rood J J MLC inhibition studies with sera from multiparous women In *Kissmeyer-Nielsen F* (Ed.) Histocompatibility Testing 1975 Munksgaard, Copenhagen, 1975 p. 594–603

Jørgensen F Lamm L U & Kissmeyer-Nielsen F Mixed lymphocyte cultures with inbred individuals An approach to MLC typing Tissue Antigens *3* 323–339 1973

Koelikarongs T., Hyttka, L., McConnachie P R. & Dossetor J B.: Serotyping for MLC gene products I Presumptive evidence that ABCIL may detect MLC factors. Tissue Antigens *5* 165–172, 1975

Lancet: B lymphocyte alloantigens in man. Lancet II 240–242 1976

van Leeuwen A., Schuit H R E. & van Rood J J.: Typing for MLC (LD) II The selection of nonstimulator cells by MLC inhibition tests using SD-identical stimulator cells (MISIS) and fluorescence antibody studies Transplant. Proc. *5* 1539–1542, 1973.

Leventhal, B. G., Buell, D., Yankee R R gentine G. N J & Terasaki, P.: The mixed leucocyte response effect of maternal plasma. In. *Harris, J E.* (Ed.) Proc. Fifth Leucocyte Culture Conference. Academic Press, New York, 1970, p. 473–486.

Lohrmann, H P., Novik s L. & Graw R. G J Cellular interactions in the proliferative response of human T and B lymphocytes to phytomitogens and allogeneic cells. J Exp. Med. *139* 1553–1567 1974

Nilsson B S Lymphocyte reactivity to PPD tuberculin. Acad. diss., Stockholm, 1972.

Revillard J P., Robert L., Betuel H., Latour M Bonneau M., Brochier J & Traeger J Inhibition of the mixed lymphocyte reaction by antibodies. Transplant. Proc *4* 173–176 1972.

Re illard, J P., Betuel H & Robert M Effects of alloantibodies on lymphocyte proliferation in itro. Cell. Immunol *8* 339–355 1973

Robert M Betuel H & Revillard, J P.: Inhibition of the mixed lymphocyte reaction by sera from multipara. Tissue Antigens *3* 39–56, 1973

an Rood J J an Leeuwen A Termijtelen A & Keuning, J J B-cell antibodies, Ia-like determinants and their relation to MLC determinants in man. Transplant. Rev *30* 122 139 1976.

an Rood J J an Leeuwen A., Keuning J J & Blussé van Oud Alblas A The serological recognition of human MLC determinants using modified cytotoxicity technique. Tissue Antigens *5* 73–79 1975

Ryhänen P Effects of anesthesia and operative surgery on the immune response of patients of different ages. Ann. Clin. Res. suppl. 19 1977

Staines, N A. & Davies D A L.: Prolongation of graft survival by anti-graft LD antibody A new pproach to donor specific immunosuppression. I *Kissmeyer-Nielsen, F* (Ed.) Histocompatibility Testing 1975, Munksgaard, Copenhagen, 1975 p. 625–628.

Thorsby E. The human major histocompatibility system. Transplant. Rev *18* 51–129 1974

Thorsby E. & Solheim, B G.: Specificity of anti-

bodies that inhibit the MLC response Transplant. Proc. *5* 1859–1862 1973
Wernet P Human Ia-type alloantigens methods of detection, aspects of chemistry and biology markers for disease states. Transplant. Rev *30* 271–298 1976
Åkerblom H Ilonen J Koivukangas T Herva E. Tiilikainen A & Kouvalainen K The value of HLA identity in the search of predisposition to juvenile diabetes mellitus. Paediat. Res. *11* 1014 1977

Acta path. microbiol. scand. Sect. C, 85: 435–440, 1977

ELIMINATION OF PROTEIN A-IgG COMPLEXES FROM THE BLOOD CIRCULATION IN RABBITS ROLE OF SPLEEN AND LIVER

R. Hällgren, G. Stålenheim and A. Bill

Departments of Internal Medicine, Medical and Physiological Chemistry and Physiology and Medical Biophysics, University of Uppsala, Sweden

Hallgren, R., Stålenheim, G. & Bill, A. Elimination of protein A-IgG complexes from the blood circulation in rabbits: role of spleen and liver. Acta path. microbiol. scand. Sect. C, 85: 435–440, 1977.

We studied the elimination from rabbit circulation of soluble immune complexes made with staphylococcal protein A and rabbit IgG. In in vivo experiments the complexes were cleared from the circulation by the liver and spleen. In the saline perfused isolated liver there was rapid uptake of protein A-IgG complexes which indicated that the elimination of these complexes by fixed tissue macrophages may occur independent of complement components.

Key words: Protein A; IgG complexes; rabbit circulation; liver; spleen.

R. Hallgren, Department of Internal Medicine, University Hospital, S-750 14 Uppsala, Sweden.

Received 11.i.77. Accepted 15.vii.77

Staphylococcal protein A (SpA), a component of the cell wall of coagulase positive staphylococci, reacts with the Fc part of IgG from all mammalian species investigated (4, 5, 6). It is known to induce a number of biological events such as the binding and activation of complement (20), anaphylaxis-like reactions (10) and phagocytosis (12). The purpose of this work was to study the disappearance and the fate of soluble circulating SpA-IgG complexes in rabbits. Similar studies of the elimination of ordinary immune complexes have been performed by other investigators (2, 16). The results of the study will be discussed in relation to the possible pathogenic role of SpA-IgG complexes in the genesis of immune complex mediated lesions associated with staphylococci infections.

MATERIAL AND METHODS

Protein A. Protein A from *Staphylococcus aureus* was prepared from lysostaphin digested bacteria by affinity chromatography (21).

Labelling of proteins. Protein A (SpA) was trace labelled with ^{125}I (the Radiochemical Centre, Amersham) as previously reported (9). Rabbit albumin (Sigma, USA) was trace labelled with ^{131}I (AB Atomenergi, Studsvik, Sweden) with 1–2 moles of iodine per mole protein, using the iodine monochloride method (17).

Preparation of ^{125}I-SpA-IgG complexes. Rabbit IgG (Cohn F. II, Miles laboratories, USA) was incubated at 4° C with ^{125}I-SpA at a ratio of 20:1 (w/w) for 24 h. Before utilization, any free iodine

and ^{125}I-SpA not reacting with IgG were removed by applying the complexes to a column of Sephadex G-200 (Pharmacia Fine Chemicals, Sweden) equilibrated with PBS pH 7 6 The ^{125}I-SpA IgG complexes appearing in the void volume were used immediately for experimental purposes.

Two series of experiments were performed

A *Albino rabbits* of both sexes and weighing 2–3 kg were employed The animals were anesthetized with i.v pentobarbital sodium, about 40 mg/kg body weight. Small additional doses were given to maintain anesthesia. Heparin 500 IU/kg body weight was given i.v The animals were kept warm with a heating pad. A femoral artery was cannulated for blood sampling 2 ml of a mixture of ^{131}I albumin and ^{125}I-SpA IgG was injected through an ear vein over a period of about 30 s The amounts of SpA and albumin were each about 0 1 mg Sequential samples of blood were taken via a femoral artery during the next 60 minutes. Ten or 60 minutes after the injection of the isotopes, the animals were killed by an intravenous injection of KCl. Samples were taken from liver spleen adrenal glands, lungs, thymus, mesenteric lymph nodes, small intestine skeletal muscle, and kidneys and immediately cooled to prevent continued catabolism of injected proteins. The amount of free iodine (^{125}I and ^{131}I) in blood and tissue samples were determined after precipitation of protein with 10 per cent trichloracetic acid.

Assay of radioactivity A two-channel gamma spectrometer was used to determine the amounts of ^{125}I and ^{131}I in the samples. Usually the counting error was less than two per cent for each isotope.

Calculations The plasma equivalent ^{125}I-SpA IgG space was calculated by dividing the amount of ^{125}I-SpA IgG (in cpm/g) recovered in the tissue by the ^{125}I-SpA IgG concentration (in cpm/µl) in the plasma using a sample taken from an ear vein just before the animal was killed. The plasma equivalent ^{131}I-albumin space was calculated in the same way

B. Albino rabbits of both sexes with a weight of 2–3 kg were used. The animals were anesthetized and heparinized as described above and ventilated using a Palmer pump The aorta was ligated above the renal artery The inferior caval vein was cannulated above the liver and then ligated near the origins of the renal veins. The portal vein at the porta hepatis was cannulated with a polyethylene tubing and the perfusion solution (Salidex, Pharmacia, Sweden) was allowed to enter the portal circulation. The animal was killed by an incision into the heart. About 600 ml was perfused through the liver at a rate of 25–30 ml/min. When the liver was blanched and empty of blood about 3 ml of a solution containing ^{131}I albumin (approximately 0 1 mg) and ^{125}I-SpA IgG (approximately 0 1 mg of SpA) was injected via the portal vein cannula. Samples of the perfusate were then collected in 8 ml portions until 250 ml of the perfusate had passed. The temperature of the perfusion solution was 23 C. One ml of each sample was tested for radioactivity as described above. After the perfusion the liver was weighed and cut into small pieces. Samples of the liver homogenate were weighed and tested for radioactivity

Calculations The concentration of ^{131}I in cpm/g of each sample of the perfusate and of the liver homogenate was divided by the concentration of ^{131}I in cpm/µl of the injection solution. This gave the apparent volume, with respect to ^{131}I of the injection solution, in µl/g, present in the effluent and the liver The data for ^{125}I were treated in a similar manner

RESULTS

Disappearance of ^{125}I-SpA IgG Complexes and ^{131}I Albumin from the Rabbit Circulation

Fig 1 shows the concentration of ^{125}I-SpA IgG and ^{131}I albumin at different times after i v injection Elimination of SpA IgG complexes was rapid compared to that of albumin The amount of free iodine appearing in the circulation is also illustrated Approximately 12 min after the injection a catabolism of ^{125}I-SpA IgG complexes was evident,

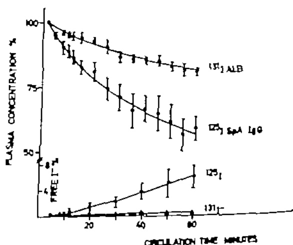


Fig 1 Mean plasma concentration of ^{125}I protein A IgG complexes and ^{131}I albumin in 5 rabbits Bars indicate S.E. Concentrations are given as the percentage of the values at two minutes after injection. The amount of free radioactive iodine is presented as the percentage of the total radioactivity in the corresponding samples.

SMALL INTESTINE
LUNG
MUSCLE
KIDNEY CORTEX
KIDNEY MEDULLA
LIVER
SPLEEN
THYMUS
LYMPH NODE
ADRENALS

10 MINUTES
60 MINUTES

0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0

RATIO 125I PROTEIN A IgG/125I ALBUMIN IN DIFFERENT TISSUES ± SEM

Fig 2 The 125I protein A-IgG/125I albumin space ratio at different times.

as indicated by the appearance of free 125I. The increase in free 125I in the circulation was almost linear from 15 to 60 min. The amounts of free 125I in blood were very low over the whole period investigated indicating that there was very little catabolism of 125I albumin.

Tissue Distribution of 125I-SpA-IgG Complexes and I-albumin

Four rabbits were killed 10 min after the injection and seven rabbits 60 min after the injection. No significant amount of either free 125I or I was detected in the organs of those animals with a circulation time of ten min. After a circulation period of 60 min however free 125I was detectable and corrected for when the tissue distribution of the radiolabelled proteins was calculated. Fig 2 show the total 125I-SpA IgG space divided by the total albumin space in the different tissue samples after circulation times of ten and sixty minutes respectively. The results indicate an uptake of SpA IgG complexes in

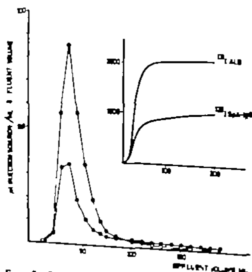


Fig 3 Elution curves for SpA-IgG complexes (dots) and 125I albumin (circles) in one of the experiments with isolated saline perfused rabbit liver. On the abscissa is given the effluent volume (ml) and on the ordinate the apparent amount of injection solution per ml effluent. The integrated elution curves are also illustrated and demonstrate marked uptake of 125I SpA-IgG complexes in the liver.

the liver and in the spleen because the ratios for these organs were greater than 1 In the other tissues investigated the ratios were less than 1 indicating a relative accumulation of ^{131}I-albumin

The Uptake of ^{125}I-SpA IgG Complexes and ^{131}I Albumin in the Saline Perfused Liver

Perfusion experiments on three rabbit livers were performed to investigate the uptake of ^{125}I-SpA IgG without the influence of plasma factors In Fig 3 data from one experiment is illustrated. There was a heavy uptake of SpA IgG complexes in relation to albumin as calculated from the integrated elution curves The accumulation of ^{125}I SpA IgG complexes was calculated as 49 40 and 32 per cent in the three livers investigated Calculations based on the recovery of ^{125}I in the liver homogenates gave almost identical uptake values of ^{125}I-SpA IgG

DISCUSSION

In the present study the elimination of SpA IgG complexes from the circulation of rabbits was compared to that of albumin. Albumin has a molecular weight of 67 000 It passes out of the capillaries in most tissues at a rather low rate In most tissues capillary permeability to still larger molecules such as IgG is less than that to albumin (3) The molecular weight of the SpA IgG complexes is about 400 000 or more as judged from the elution pattern after gel separation The rapid elimination of SpA IgG complexes from the blood indicates an uptake in some tissues and the results of the tissue essays indicates that the uptake occurred in the liver and spleen. It was expected to observe SpA IgG/albumin space ratios of < 1.0 in most tissues and ratios > 1.0 indicate an accumulation mechanism for the complexes. These results are in agreement with those of previous studies on ordinary immune complexes (16)

Cells of the phagocytic system recognize immune complexes and eliminate them by adherence and subsequent phagocytosis The process of recognition is at least in part mediated via specific membrane receptors for IgG (15) and the third activated component of the complement system (13) The role of plasma factors in the phagocytic uptake of HSA anti HSA complexes has been analysed in a rabbit system by other investigators Depletion of complement by aggregated IgG or the anti-complementary cobra venom factor (2) had no effect on the rate of disappearance of the complexes from the circulation.

In order to determine if complement is necessary for the elimination of SpA IgG complexes we perfused rabbit liver to blood emptiness and studied the uptake without the presence of plasma factors. Although the participation of interstitial or cell-bound complement components is not rigorously ruled out by this approach, it seems less likely that complement activation occurred especially as SpA IgG complexes in a rabbit system poorly activate the complement system (22)

Our results thus indicate that the uptake in the liver of SpA IgG complexes does not require the presence of complement or other plasma proteins. Since as much as 30–40 per cent of the complexes were entrapped during a single perfusion in a plasma free liver the elimination rate *in vivo* seems unexpectedly low

In view of the importance of an intact Fc part of IgG for ingestion by phagocytes (2) the binding of SpA to the Fc might be a hindrance to the union of the SpA IgG complex and the Fc receptor on the cell membrane. The present data, which are in agreement with our previous *in vitro* studies of phagocytosis of SpA IgG complexes (7) are interpreted to mean that the binding of SpA to the Fc piece does not prevent its reaction with the Fc reactive sites of cells.

This investigation indicates that protein A IgG complexes provide an experimental model well suited for the study of the elimination of immune complexes. The model has certain advantages which deserve enumeration. Immunization of animals with heterologous serum proteins gives different degrees of antibody response which influences the

ure and, as a consequence, also the fate of immunecomplexes formed after injection of serum protein antigens (1) In contrast, it is easy to standardize the formation of protein A-IgG complexes as the affinity between protein A and IgG is constant and the amount of IgG in circulation in each species varies moderately Moreover there is no need for prior immunization of the experimental animal.

The relationship between the present findings and the proposed role of protein A as a pathophysiological agent in immune complex mediated lesions in man is not clear Acute glomerulonephritis has been associated with *Staphylococcus aureus* infections (23) and the focal deposition of IgG and complement in the glomeruli has indicated an immune complex nephritis (18) Bacterial agents have not been demonstrated in the glomeruli, but *Kronvall & Gewurz* (14) have suggested that the glomerular lesions might be due to the appearance of protein A-IgG complexes. Most pathogenic staphylococci contain protein A in the cell wall, and some strains release newly synthetized protein A extracellularly (7 19) During a severe staphylococcal infection, a large amount of protein A may thus appear in the circulation. The present results indicate that protein A in complex with IgG is normally eliminated via RES but may circulate for many hours. Dextran molecules with about the same molecular weight as SpA IgG complexes are known to pass through the capillary wall in several tissues (8, 1) Thus especially in situations with impaired RES capacity circulating SpA IgG complexes are likely to pass through the capillary walls and deposite in connective tissue causing inflammation.

This study was supported by the *Swedish Medical Research Council* (Grant B0-15X 2535-02) and the *King Gustaf V 80-years foundation*.

REFERENCES

1 *Alpers J H., Steward M W & Soothill, J F.:* Differences in immune elimination in inbred mice. The role of low affinity antibody Clin. Exp. Immunol. *I* 121 132, 1972.

2. *Arend W P & Mannik M.,* II Quantification of tissue uptake of sol ble complexes in normal and complement depleted rabbits. J Immunol. *107* 63–75, 1971

3 *Bill A* Plasma protein dynamics albumin nd IgG capillary permeability extravascular movement and regional blood flow in unanesthetized rabbits. In press. Acta Physiol Scand

4 *Forsgren, A* Protein A from *Staphylococcus au eus V* Reaction with guinea pig γ-globulins. J Immunol. *100* 921–926, 1968.

5 *Forsgren A & Sjöquist J* Protein A" from *S au eus* I Pseudo-immune reaction with human γ-globulin. J Immunol. *97* 822–827 1966.

6. *Forsgren A & Sjöquist J* Protein A from *Staphylococcus aureus* III Reaction with rabbit γ-globulin. J Immunol. 99 19–24 1967

7 *Forsgren A.* Protein A from *Staphylococcus aureus* VIII. Production of protein A by bacterial and L-forms of *S aureus* Acta path. microbiol. scand. Sect. *75* 481–490 1969

8. *Garlick D G & Renkin E M* Transport of large molecules from plasma to interstitial fluid and lymph in dogs Am. J Physiol. *219* 1596–1605 1970.

9 *Ghetie V Stålenheim G & Sjöquist J* Cell separation by staphylococcal protein A coated erythrocytes Scand. J Immunol. *4* 471-477 1975.

10. *Gustafsson G T Stålenheim G Forsgren A & Sjöquist J* "Protein A from *Staphylococcus aureus* IV Production of anaphylaxis-lik cutaneous and systemic reactions in nonimmunized guinea-pig. J Immunol. *100* 530–534 1968.

11 *Grotte G* Passage of dextran molecules across the blood-lymph barrier Acta Chir Scand. Suppl. 211 1956.

12. *Hällgren, R. & Stålenheim G* Quantification of phagocytosis by human neutrophils. The use of radiolabelled Staphylococcal Protein A-IgG complexes. Immunol. *30* 755–761 1976.

13 *Huber H Polley M J Linscott W J Fudenberg H H & Müller-Eberhard H J* Human monocytes distinct receptor sites for the third component of complement and for immunoglobulin G Science (Wash. D.C.) *162* 1281–1283 1968.

14 *Kronvall, G & Gewurz H* Activation and inhibition of IgG mediated complement fixation by staphylococcal protein A. Clin Exp. Immunol. *7*2 211–220, 1970

15. *Le Buglio A F., Cotran R.S & Jandl J H* Red cells coated with immunoglobulin G binding and sphering by mononuclear cells in

the liver and in the spleen because the ratios for these organs were greater than 1 In the other tissues investigated the ratios were less than 1 indicating a relative accumulation of ^{131}I albumin

The Uptake of ^{125}I-SpA IgG Complexes and ^{131}I Albumin in the Saline Perfused Liver

Perfusion experiments on three rabbit livers were performed to investigate the uptake of ^{125}I-SpA IgG without the influence of plasma factors. In Fig 3 data from one experiment is illustrated. There was a heavy uptake of SpA IgG complexes in relation to albumin as calculated from the integrated elution curves The accumulation of ^{125}I SpA IgG complexes was calculated as 49 40 and 32 per cent in the three livers investigated Calculations based on the recovery of ^{125}I in the liver homogenates gave almost identical uptake values of ^{125}I-SpA IgG

DISCUSSION

In the present study the elimination of SpA IgG complexes from the circulation of rabbits was compared to that of albumin. Albumin has a molecular weight of 67 000 It passes out of the capillaries in most tissues at a rather low rate. In most tissues capillary permeability to still larger molecules such as IgG is less than that to albumin (3) The molecular weight of the SpA IgG complexes is about 400 000 or more as judged from the elution pattern after gel separation The rapid elimination of SpA IgG complexes from the blood indicates an uptake in some tissues and the results of the tissue essays indicates that the uptake occurred in the liver and spleen It was expected to observe SpA IgG/albumin space ratios of < 1.0 in most tissues and ratios > 1.0 indicate an accumulation mechanism for the complexes These results are in agreement with those of previous studies on ordinary immune complexes (16)

Cells of the phagocytic system recognize immune complexes and eliminate them by adherence and subsequent phagocytosis The process of recognition is at least in part mediated via specific membrane receptors for IgG (15) and the third activated component of the complement system (13) The role of plasma factors in the phagocytic uptake of HSA-anti HSA complexes has been analysed in a rabbit system by other investigators Depletion of complement by aggregated IgG or the anti-complementary cobra venom factor (2) had no effect on the rate of disappearance of the complexes from the circulation.

In order to determine if complement is necessary for the elimination of SpA IgG complexes we perfused rabbit liver to blood emptiness and studied the uptake without the presence of plasma factors. Although the participation of interstitial or cell bound complement components is not rigorously ruled out by this approach it seems less likely that complement activation occurred especially as SpA IgG complexes in a rabbit system poorly activate the complement system (22)

Our results thus indicate that the uptake in the liver of SpA IgG complexes does not require the presence of complement or other plasma proteins. Since as much as 30–40 per cent of the complexes were entrapped during a single perfusion in a plasma free liver the elimination rate *in vivo* seems unexpectedly low

In view of the importance of an intact Fc part of IgG for ingestion by phagocytes (2) the binding of SpA to the Fc might be a hindrance to the union of the SpA IgG complex and the Fc receptor on the cell membrane. The present data which are in agreement with our previous *in vitro* studies of phagocytosis of SpA IgG complexes (7) are interpreted to mean that the binding of SpA to the Fc piece does not prevent its reaction with the Fc reactive sites of cells

This investigation indicates that protein A IgG complexes provide an experimental model well suited for the study of the elimination of immune complexes. The model has certain advantages which deserve enumeration. Immunization of animals with heterologous serum proteins gives different degrees of antibody response which influences the

ure and, as a consequence, also the fate of immunecomplexes formed after injection of serum protein antigens (1) In contrast, it is easy to standardize the formation of protein A-IgG complexes as the affinity between protein A and IgG is constant and the amount of IgG in circulation in each species varies moderately Moreover there is no need for prior immunization of the experimental animal.

The relationship between the present findings and the proposed role of protein A as a pathophysiological agent in immune complex mediated lesions in man is not clear Acute glomerulonephritis has been associated with *Staphylococcus aureus* infections (23) and the focal deposition of IgG and complement in the glomeruli has indicated an immune complex nephritis (18) Bacterial agents have not been demonstrated in the glomeruli, but *Kronvall & Gewurz* (14) have suggested that the glomerular lesions might be due to the appearance of protein A IgG complexes. Most pathogenic staphylococci contain protein A in the cell wall, and some strains release newly synthetized protein A extracellularly (7 19) During a severe staphylococcal infection, a large amount of protein A may thus appear in the circulation. The present results indicate that protein A in complex with IgG is normally eliminated via RES, but may circulate for many hours. Dextran molecules with about the same molecular weight as SpA IgG complexes are known to pass through the capillary wall in several tissues (8, 1) Thus especially in situations with impaired RES capacity circulating SpA IgG complexes are likely to pass through the capillary walls and deposite in connective tissue causing inflammation.

This study was supported by the *Swedish Medical Research Council* (Grant B0-14X-2533-02) and the *King Gustaf V 80-years foundation*.

REFERENCES

1 *Alpers J H Steward M W & Soothill, J F* Differences in immune elimination in inbred mice The role of low affinity antibody Clin. Exp. Immunol. *12:* 121–132, 1972.

2. *Arend W P & Mannik, M.* II Quantification of tissue uptake of soluble complexes in normal and complement depleted rabbits. J Immunol. *107* 63–73 1971

3 *Bill A.:* Plasma protein dynamics albumin and IgG apillary permeability extravascular movement and regional blood flow in unanesthetized rabbits. In press. Acta Physiol. Scand.

4 *Forsgren A.:* Protein A from *Staphylococcus aureus* I Reaction with guinea pig γ-globulins. J Immunol. *100* 921 926, 1968

5 *Forsgren, A & Sjöquist J* Protein A" from *S aureus.* I Pseudo-immune reaction with human γ-globulin. J Immunol. *97* 822–827 1966.

6. *Forsgren A. & Sjöquist J* Protein A from *Staphylococcus aureus* III. Reaction with rabbit γ-globulin. J Immunol. *99* 19–24 1967

7 *Forsgren A* Protein A from *Staphylococcus aureus* VIII Production of protein A by bacterial and L-forms of *S aureus,* Acta path. microbiol. scand. Sect. *75* 481–490 1969

8. *Garlick D G. & Renkin, E. M.* Transport of large molecules from plasma to interstitial fluid and lymph in dogs. Am. J Physiol. *219* 1596–1605, 1970

9 *Ghetie V., Stålenheim G. & Sjöquist J.:* Cell separation by staphylococcal protein A coated erythrocytes. Scand. J Immunol. *4* 471–472 1975

10. *Gustafsson G. T., Stålenheim G., Forsgren A & Sjöquist J* "Protein A from *Staphylococcus aureus* IV Production of anaphylaxis-like cutaneous and systemic reactions in nonimmunized guinea-pig. J Immunol. *100* 530-534 1968.

11 *Grotte G* Passage of dextran molecules across the blood-lymph barrier Acta Chir Scand. Suppl. 211 1956.

12. *Hällgren R. & Stålenheim G* Quantification of phagocytosis by human neutrophils. The use of radiolabelled Staphylococcal Protein A-IgG complexes. Immunol. *30* 755-761 1976

13 *Huber H Polley M J., Linscott W J Fudenberg H H & Müller-Eberhard H J* Human monocytes distinct receptor sites for the third component of complement and for immunoglobulin G Science (Wash. D.C.) *162* 1281–1283 1968

14 *Kronvall G. & Gewurz, H* Activation and inhibition of IgG mediated complement fixation by staphylococcal protein A. Clin. Exp. Immunol. *7* 211 220 1970

15 *Lo Buglio A F Cotran R.S & Jandl J H.:* Red cells coated with immunoglobulin G binding and sphering by mononuclear cells in

man. Science (Wash. D C.) *158* 1582–1585 1967

16 *Mannik M & Arend W P* Fate of preformed immune complexes in rabbits and rhesus monkeys. J Exp Med *134* 19–31 1971

17 *Mc Farlane A S* Efficient tracer labelling of proteins with iodine. Nature (Lond.) *182* 53–55 1958

18 *Michael A F Westberg N G Fish A J & Vernier R L.* Studies on chronic membranoproliferative glomerulonephritis with hypocomplementia. J Exp Med. *134* 208–227, 1971

19 Movitz, J Formation of extracellular protein A by *Staphylococcus aureus* Eur J Biochem. *68* 291–299 1976

20 *Sjöquist J & Stålenheim G.,* Protein A from *Staphylococcus aureus* IX. Complement fixing activity of protein A IgG complexes. J Immunol. *103* 467–473 1969

21 *Sjöquist J Meloun B & Hjelm H.,* Protein A isolated from *Staphylococcus aureus* after digestion with lysostaphin. Eur J Biochem. *29* 572–578 1972.

22 *Stålenheim G* Protein A from *Staphylococcus aureus* XI Fixation of human complement and complement from guinea pig and rabbit. Acta path. microbiol. scand. Sect. B *79* 665–672 1971

23 *Tu W Shearn M A & Lee J* Acute diffuse glomerulonephritis in acute staphylococcal endocarditis. Ann. Intern. Med. *71* 335–341 1969

Acta path. microbiol. scand. Sect. B, 85: 441–448, 1977

ISOLATION AND IMMUNOCHEMICAL DETERMINATION OF SOW COLOSTRUM TRYPSIN INHIBITOR

P. Thode Jensen

The State Veterinary Serum Laboratory
Bülowsvej 27, DK 1870 Copenhagen V, Denmark

Thode Jensen, P. Isolation and immunochemical determination of sow colostrum trypsin inhibitor. Acta path. microbiol. scand. Sect. C, 85: 441–448, 1977.

Trypsin inhibitor from sow colostrum was isolated by ion exchange chromatography on DEAE-Sephadex A 50 followed by gel filtration chromatography on Sephadex G-100 and affinity chromatography. Antiserum against sow colostrum trypsin inhibitor was produced by immunization with the purified inhibitor and made specific by absorption with normal porcine serum. The specific antiserum was used for immunoquantitation by single radial immunodiffusion (SRI). In sow colostrum whey good agreement was found between the results obtained by SRI and the total trypsin-inhibiting activity as determined by radial diffusion in casein-containing agarose gel (r = 0.97, n = 10). In sow's milk there was only a very low inhibiting activity and no colostral inhibitor was demonstrable by SRI. Also in baby-pig urine agreement was found between the two methods (r = 0.97, n = 14). In baby-pig serum such an agreement was not seen, undoubtedly because of the presence of genuine serum trypsin inhibitors. By the SRI technique it is possible specifically to determine the colostral inhibitor even in the presence of other trypsin inhibitors.

Key words: Sow colostrum trypsin inhibitor; trypsin inhibitor immunoquantitation; affinity chromatography.

P. Thode Jensen, The State Veterinary Serum Laboratory,
Bülowsvej 27, DK-1870 Copenhagen V, Denmark

Received 14.iv.77 Accepted 15.vi.77

A trypsin inhibitor specific for porcine colostrum and with a high degree of resistance to pepsin and acid was first isolated and described by *Laskowski et al.* (1957). The inhibitor concentration in colostrum was highest on the first day after parturition and fell gradually to about zero by the fifth day. The colostral inhibitor is assumed to protect maternal antibodies during absorption from the gut (*Barntner* 1973), and was claimed (*Barntner* 1970) to be excreted in the urine of the newborn piglets shortly after colostrum ingestion. This latter supposition agrees with the findings of *Carlsson & Karlsson* (1972, 1973) and *Carlsson et al.* (1974). Gel filtration studies by *Carlsson et al.* revealed a molecular weight of about 18,000 for the colostral inhibitor and about 70 000 for serum inhibitors. By preparative electrophoresis of sow colostrum *Carlsson & Karlsson* (1973) found the trypsin-inhibiting activity to be located in the γ-region. After concentration of the electrophoretic fractions a weak in-

hibiting activity was found also in those fractions which had a migration rate corresponding to the serum inhibitors (α region).

All these investigations were made by measuring the trypsin inhibiting capacity of the samples by means of different substrates. By such methods it is not possible to differentiate the colostral inhibitor from other trypsin inhibitors. The aim of the present investigation was to isolate sow colostrum trypsin inhibitor and to work out a specific immunochemical method for measuring it in biological fluids.

MATERIALS AND METHODS

Porcine colostrum for isolation of trypsin inhibitor was collected from 10 Danish landrace sows immediately after parturition. *Sow's milk* was collected 20–40 days after parturition. *Blood and urine samples from neonatal piglets* were collected at intervals during the period of colostrum feeding. Blood was obtained by puncture of the anterior vena cava, and urine by vesicocentesis. All samples were frozen as soon as possible and stored at −20° C until used.

Preparation of colostral and milk whey partly freed from β lipoproteins. Sow colostrum was thawed, pooled, and centrifuged at 18,000 *g* for one hour at 4° C. The fat layer at the top and the pellet at the bottom were discarded. Colostrum whey was produced from the de-fatted colostrum according to the method of *Aalund* (1968) and used for the preparation of trypsin inhibitor after precipitation of β-lipoproteins by the method of *Burstein (1960). This treatment, which served to* reduce the viscosity of the whey before chromatography, had no effect on the trypsin inhibiting capacity.

Chromatographic Methods

Ion exchange chromatography with step-wise elution was performed at 4° C on DEAE-Sephadex A 50 (Pharmacia, Sweden). A 0.1 M tris-HCl 0.2 mM $CaCl_2$ buffer pH 8.2 was used for swelling the gel and for initial elution. The same buffer with addition of NaCl (0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M, and 0.6 M) was used for the step-wise elution. The protein content in the effluent from the chromatography column (5×30 cm) was monitored continuously at 280 nm on an LKB 8300 Uvicord (LKB-product AB, Sweden).

Gel filtration chromatography was performed at 4° C on a 2.5×60 cm column of Sephadex G 100 (Pharmacia, Sweden) in 0.1 M tris-HCl 0.2 mM $CaCl_2$ buffer pH 8.2, and on a 1.5×30 cm column of *Sephadex G-75* with the same buffer. A constant upward flow of 12 ml/h was maintained and the protein concentration in the effluent was recorded as described above. Concentration of the chromatographic fractions was made by vacuum dialysis.

Affinity chromatography was performed according to *Chauvet & Acher* (1972) with some modifications. Five grams of cyanogen bromide activated Sepharose 4 B (Pharmacia, Sweden) was washed and reswelled with 1 l 1 M HCl on a sintered-glass filter (G 3) and then rapidly washed with about 0.5 l of cold 0.1 M $NaHCO_3$ 0.5 M NaCl buffer pH 9.0. The activated Sepharose was suspended in 20 ml of the same buffer containing 200 mg porcine trypsin ("Trypure" Novo, Denmark) and gently stirred at room temperature overnight. The trypsin-Sepharose was washed with 1 l 0.1 M $NaHCO_3$ 0.5 M NaCl buffer pH 9.0, *resuspended in 0.5 M ethanolamine pH 9.0 and* gently stirred at 4° C overnight. The trypsin-coupled gel was washed five times with, alternately, a 0.1 M acetate 0.5 M NaCl 0.01 M $CaCl_2$ *buffer pH 4.0 and a 1 M NaCl solution, and then* transferred to a column of 1.5×15 cm. The column was washed with 200 ml 0.1 M HCl 0.5 M NaCl 0.01 M $CaCl_2$ solution at room temperature and subsequently equilibrated with the acetate buffer. The partially purified colostral inhibitor was dialysed against the acetate buffer and applied to the column, which was left overnight at room temperature. The column was then washed with the acetate buffer until no more protein could be measured in the effluent. After that it was washed with 0.5 M NaCl solution and again with the acetate buffer. Inhibitor was eluted by a 0.1 M HCl 0.5 M NaCl 0.01 M $CaCl_2$ solution. The effluent was collected in tubes containing 0.5 M tris-HCl buffer pH 8.5 for neutralizing the acid. The active fractions were pooled, concentrated, and dialysed against a 0.1 M tris-HCl 0.2 mM $CaCl_2$ 15 mM NaN_3 buffer pH 8.2 and stored at +4° C.

Determination of Activity

Radial diffusion assay for semiquantitative evaluation of trypsin-inhibiting activity in colostrum samples and chromatographic fractions was performed according to the method of *Carlsson & Karlsson* (1972) using casein as a substrate.

Quantitative determination of the trypsin-inhibiting activity was performed by the radial diffusion technique just referred to, though with the following modifications. After the casein agarose had solidified, wells with a diameter of 4 mm were punched in the gel (Fig. 1). Of each sample a series of two-fold dilutions in saline were prepared in tubes. Each dilution was mixed with an equal volume of a saline solution of trypsin (50 μg/ml)

hereafter 15 μl of each mixture was placed in a well. After incubation for 5 hours in a humid atmosphere at 37 C the plates were fixed and dried. Staining was performed for 5 min in solution of Coomassie Brilliant Blue R. Trypsin activity would manifest itself as circular unstained zones around the wells, and, conversely, absence of such zones would indicate inhibition of the trypsin (Fig. 1). Inhibiting activity was expressed by the amount of trypsin inhibited per ml (μg trypsin inhibited/ml sample). Values between those pertaining to two consecutive sample dilutions were obtained by interpolation between the areas of the zones concerned. In serial dilutions with more than one dilution giving a partially blocked zone the inhibitor concentrations were expressed by the lowest amount of trypsin used per ml sample to give less than 5 per cent inhibition.

Immunoelectrophoretic Methods

The equipment and reagents for immunoelectrophoresis were essentially as described by *Weeke* (1973 a). The electrophoresis was carried out in 1 per cent agarose (w/) (Indubiose A 37 L'Industrie Biologique Française S.A.) in 0.016 M Barbital-N 0.003 M Barbital 0.003 M sodium azide pH 8.6. The same buffer four times concentrated as used in the electrophoresis vessels. Rocket immunoelectrophoresis and line immunoelectrophoresis were performed as described by *Weeke* (1973b) and *Krøll* (1973). The electrophoresis were performed in 0.1 cm thick gel on 10 × 10 cm glass slides at 2 V/cm for 18 hours. After completion of the electrophoresis process the plates were pressed, washed, dried, and finally stained with Coomassie Brilliant Blue R.

Single radial immunodiffusion (SRI) for immunoquantitation (*Mancini et al.* 1965) was made in the same agarose gel plates as described above, supplemented with polyethylene glycol 6000 to a concentration of 2.5 per cent. Purified colostral trypsin inhibitor and pool of sow colostrum (n = 10) were used for reference.

Preparation of Antisera

Antisera were prepared by immunization of rabbits according to the method of *Harboe & Ingild* (1973). T each of 8 rabbits, at least 3 intradermal injections with 50 μg purified trypsin inhibitor were given at intervals of 2 weeks. The antisera were absorbed with pool of adult porcine normal serum and subsequently tested by rocket and line immunoelectrophoresis against sow colostrum.

Protein determination. Total protein was determined by the method of *Lowry et al.* (1951) using bovine serum albumin as standard.

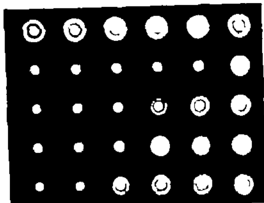


Fig. 1. Radial diffusion assay in casein-containing agarose gel (150 μg casein/cm). Fifteen μl of trypsin-urine mixture was applied to each hole. From the left the urine concentrations were 1/2 – 1/4 – 1/8 – 1/16 – 1/32 – 1/64. The trypsin concentration was 25 μg/ml in all holes. Unhibited trypsin activity in all holes of the top row.

RESULTS

Purification of Colostral Trypsin Inhibitor

By ion exchange chromatography on colostrum whey fractions with strong trypsin-inhibiting activity were obtained only in the descending part of the fall-through peak. These fractions were pooled, concentrated, and gel filtered on Sephadex G-100. The elution pattern is shown in Fig. 2 A. After rechromatography on Sephadex G-100 the fractions with strong inhibiting activity were further purified by affinity chromatography and used for immunization. The elution zones with trypsin inhibiting activity from concentrated fractions obtained by Sephadex G-100 chromatography of whole colostrum whey are indicated in Fig. 2 B.

Immunochemical Determination of Colostral Trypsin Inhibitor

From just one of eight immunized rabbits was a serum obtained which gave distinct precipitation lines when tested by immunoelectrophoresis against the immunization material, i.e., purified trypsin inhibitor or against sow colostrum and porcine serum. With colostrum whey this antiserum gave one

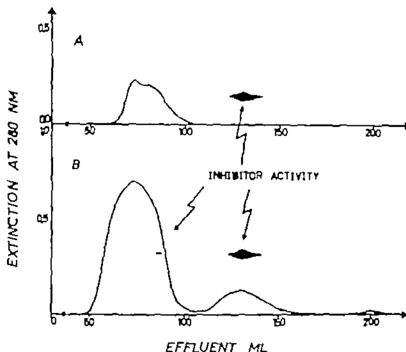


Fig 2 Elution diagrams from Sephadex G-100 of (A) Pooled concentrated trypsin-inhibiting fractions from DEAE-Sephadex A-50 chromatography of sow-colostrum whey (B) Sow-colostrum whey

Elution pattern of trypsin inhibitor was determined by radial diffusion assay in casein-containing agarose gel

TABLE 1 *Trypsin Inhibitor Concentrations in Sow Colostrum Whey and Sow's Milk (3-6 Weeks after Parturition) as Measured by Radial Diffusion Assay in Casein-containing Agarose (RDA) and by Single Radial Immunodiffusion (SRI)*

Sow No.	Sow colostrum whey RDA mg trypsin inhibited / ml sample	Sow colostrum whey SRI µg / ml sample	Sow's milk RDA µg trypsin inhibited / ml sample	Sow's milk SRI µg / ml sample
1	1.30	550	< 10	NM
3	1.50	626	< 10	NM
36	0 90	332	< 10	NM
38	0 85	373	< 10	NM
39	1 60	314	< 10	NM
56	1.90	673	< 10	NM
99	3.55	1236	< 10	NM
179	1 90	632	< 10	NM
181	2 05	639	< 10	NM
295	2 15	858	< 10	NM

NM = not measurable

distinct and one faint line With the purified colostral trypsin inhibitor used for immunization it gave one strong and one weak line and with porcine serum one weak line Partial identity was found between the distinct colostrum line and the strong line developed with the purified inhibitor furthermore the spur from the colostrum precipitate showed identity reaction with the weak line of the purified inhibitor (Fig 3) A pattern of identity was also seen between the faint colostrum line and the serum line Specificity for sow colostrum inhibitor was obtained after absorption of the antiserum with appropriate amounts of porcine normal serum (Fig 4) The distinct precipitate was iden

Fig 3 Line immunoelectrophoretic comparison of A. 5 µl colostrum whey B. 5 µl purified sow colostrum trypsin inhibitor (85 µg/ml) C. 5 µl purified sow colostrum trypsin inhibitor (30 µg/ml) D. 5 µl porcine serum pool. E. 5 µl colostrum whey. The antibody gel contained antiserum against purified sow colostrum trypsin inhibitor 10 µl/cm². Anode at the top.

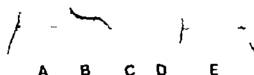


Fig 4 Line immunoelectrophoretic comparison of A. 15 µl colostrum whey B. 15 µl purified sow colostrum trypsin inhibitor (85 µg/ml) C. 15 µl baby pig urine D. 15 µl porcine serum pool. The antibody gel contained antiserum against purified sow colostrum trypsin inhibitor after absorption with 10 per cent (v/v) porcine serum pool, 15 µl/cm². Anode at the top.

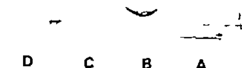
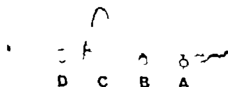


Fig 5 Rocket immunoelectrophoresis of two different colostrum samples (A, B) purified sow colostrum trypsin inhibitor (C) and porcine serum pool D) The samples were applied under weak current. The antibody gel contained antiserum against purified sow colostrum trypsin inhibitor after absorption with 10 per cent (v/v) porcine serum pool 15 µl/cm². Anode at the top. Note the side diffusion and the cathodic movement of the precipitate.



tified as an inhibitor-antiinhibitor complex on account of the agreement found between the results obtained on examination of colostrum and milk by respectively single radial immunodiffusion and radial diffusion assay (RDA) (Table 1). A correlation coefficient of 0.97 (n = 10) was found.

The results of comparative examinations on serum and urine from neonatal piglets at different intervals after their first colostrum meal are given in Table 2. There was good agreement between the results obtained by the two methods as far as the urine samples are concerned (r = 0.97 n = 14) but discrepancy regarding the serum samples.

As seen from Fig 5 there was a tendency to "side diffusion" of the inhibitor and cathodic movement of the precipitate during the electrophoretic runs. This was also observed after application of the samples under a weak current. Of the inhibitor fractions represented in Fig. 2, only those from the low mol. wt. zone showed reaction with the specific antiinhibitor. By gel filtration of the

TABLE 2 *Trypsin Inhibitor Concentrations in Serum and Neonatal Piglets (I–V) at Different Intervals (Hours) after Their First Colostrum Meal. The Concentrations Were Measured by Radial Diffusion Assay in Casein-containing Agarose (RDA) and by Single Radial Immunodiffusion (SRI)*

Pig No. hours	Serum RDA mg trypsin inhibited / ml sample	Serum SRI µg / ml sample	Urine RDA µg trypsin inhibited / ml sample	Urine SRI µg / ml sample
I – 5	1 6	59	1109	535
II – 5	3.2	51	845	548
III – 5	3.2	61	400/	1062
IV – 5	6 4	49	916	449
V – 5	3 2	40	479	190
I – 28	1 6	3	154	82
II – 28	3 2	NM	50	25
III – 28	6 4	NM	56	23
IV – 28	3.2	NM	38	14
V – 28	3 °	4	258	115
I – 51	3 2	NM	ND	ND
II – 51	3.2	NM	24	3
III – 51	3 °	NM	11	< 1
IV – 51	3.2	NM	11	< 1
V – 51	3 2	NM	5	2

) Lowest amount of trypsin used per ml sample to give less than 5 per cent inhibition.
NM = not measurable *ND* = not done.

purified colostral trypsin inhibitor on Sephadex G 75 *no further separation according* to mol. wt. was accomplished.

DISCUSSION

The present paper describes an immunochemical method for determination of sow colostrum trypsin inhibitor independently of all other inhibitors This is not possible with methods based on trypsin inhibiting capacity The acid stability of the trypsin inhibitor (*Laskowski et al 1957*) permitted the use of the affinity chromatography method of *Chauvet* & *Acher* (1972) for its final purification including an elution procedure with hydroclonc acid as the elution agent. By this procedure it was easy to break the bindings between the trypsin inhibitor and porcine trypsin covalently bound to the Sepharose matrix, a problem which had been found difficult in some preliminary investigations. Gel filtration on Sephadex G-100 before affinity chromatography *helps separating the* specific colostrum inhibitor (mol. wt. 18,000) *from small amounts of other inhibitors, e.g.,* serum inhibitors (mol. wt. about 70 000) which can be found also in colostrum (*Carlsson et al.* 1974 *Carlsson* & *Karlsson* 1973) Nevertheless a faint immunoprecipitate was found when rabbit natisera raised *against purified colostral inhibitor* were tested against adult normal porcine serum This precipiting antigen was found to be immunologically identical with an antigen in colostrum. No attempt was made to identify this precipitate it may be due to antibodies induced by small amounts of an unidentified trypsin inhibitor contaminating the purified colostral inhibitor preparation used as antigen, and occurring in adult pig serum too, or to some other protein unspecifically bound to Sepharose and eluted together with the inhibitor

Low molecular weight and slow electrophoretic velocity was described by *Weeke* (*1973 b*) as a cause of "side diffusion" of *proteins* during *rocket* immunoelectrophore-

es *Carlsson* & *Karlsson* (1973) using gel filtration studies, demonstrated that the colostral trypsin inhibitor had a mol. wt. of about 18,000, and by agarose gel electrophoresis *Carlsson et al* (1974) found it to be placed in the γ-globulin region. These circumstances will probably explain the "wide diffusion" of the inhibitor and the tendency to cathodic movement of the precipitate.

The double precipitate (Fig. 3 and 4) formed by the purified colostral inhibitor is assumed to be an artefact caused by partial proteolytic degradation during purification. In immunoelectrophoresis of erythrocyte membrane proteins artefacts were described by *Bjerrum* & *Bøg-Hansen* (1975) to be caused by leucocyte proteases contaminating the erythrocyte preparations. Also in colostrum-protein preparations there is a risk of contamination with leukocytes and leukocyte proteases, which may be able to degrade the inhibitor during the separation procedure. The little peak of trypsin-inhibiting activity found, after concentration, in some early fractions from the Sephadex G 100 filtration of colostrum whey (Fig. 2) was probably caused by the same inhibitor as found by *Carlsson* & *Karlsson* (1973) in the α-globulin region after pevicon electrophoresis of sow colostrum. This conclusion is supported by the fact that these fractions did not react immunochemically with antiserum for colostral inhibitor. The discrepancy between the results obtained on examination of sera from suckled neonatal piglets by respectively radial diffusion assay and SRI is presumably due to the strong inhibiting activity shown by *Carlsson* & *Karlsson* (1972, 1973) and *Carlsson et al* (1974) to be an inherent feature of foetal and neonatal pig serum. The occurrence, in RDA, of more than one partially blocked zone in a series of dilutions was associated with serum samples only and should probably be ascribed to differences between the binding constants for serum and colostral trypsin inhibitors. There was a fine agreement between the results of the two methods when applied to urine from the same piglets, which confirms that only the colostral inhibitor is eliminated in the urine. The quantitative immunochemical determinations of colostral inhibitor in serum and urine from neonatal piglets verify the conclusions by *Bäintner* (1970) and *Carlsson* & *Karlsson* (1972, 1973) that in neonatal piglets the colostral inhibitor is very efficiently eliminated by the kidneys, in that the low concentration of the colostral inhibitor in serum from suckled neonatal piglets as compared to the high concentration in the urine implies a very quick elimination. This agrees with the finding by *Carlsson et al* (1974) that on intravenous injection of colostrum into 10-day old piglets most of the trypsin inhibiting activity appeared in the urine within a few hours of the injection.

By the immunochemical method described, a specific, quantitative determination of sow colostrum trypsin inhibitor in biological fluid is easily performed.

The author is pleased to acknowledge the skillful technical assistance of Mrs *B. Lüders J nsen* and Mrs *Lisbeke Sørensen*. Thanks are also due to Dr *C. Wolstrup* who carried out the total protein determinations, and to Mrs *A. M. Ostergaard*, Mr *F. Larsen* and Mr *P. Thomassen* who made the photographs.

REFERENCES

Aalund O: Heterogeneity of ruminant immunoglobulins. Thesis. Munksgaard, Copenhagen 1968.

Bäintner J K: Urinary excretion of colostral trypsin inhibitor in neonatal piglets. Life Sci. 9: 847–849, 1970.

Bäintner J K: The physiological role of colostral trypsin inhibitor: experiments with piglets and kittens. Acta Vet. Acad. Sci. Hung. 23: 247–260, 1973.

Bjerrum O J & *Bøg-Hansen T C*: Analysis of partially degraded proteins by quantitative immunoelectrophoresis. In: *Axelsen N H* (Ed.): Quantitative immunoelectrophoresis. New developments and applications. Scand. J. Immunol. 4, Suppl. 2: 89–99, 1975.

Burstein M: Dosage du cholestérol dans les α et les β lipoprotéines du sérum par une méthode basée sur la précipitation sélective des β lipoprotéines. Path. Biol. 8: 1247–1249, 1960.

Carlsson L C T & *Karlsson B W*: Trypsin in-

hibiting capacity in serum digestive contents, urine and organs of the developing pig Enzymologia *43* 8–103 1972.

Carlsson L. C T & Karlsson B W Electrophoretically separable trypsin inhibitors of the developing pig Enzyme *14* 261–274 1973

Carlsson L. C T Bergelin I S S & Karlsson B W Trypsin inhibition in urine of developing neonatal pigs and in sow's colostrum. Enzyme *18* 176–188 1974

Chauvet J & Acher R Isolation of trypsin inhibitor (Kunitz inhibitor) from bovine ovary by affinity chromatography through trypsin sepharose. FEBS Letters *23* 317–320 1972

Harboe N & Ingild A Immunization, isolation of immunoglobulins, estimation of antibody titre In *Axelsen N H Krøll J & Weeke B* (Eds.) A manual of quantitative immunoelectrophoresis Methods and applications. Scand J Immunol *2* Suppl. 1 161–164 1973

Krøll, J Line immunoelectrophoresis. In *Axelsen N H Krøll J & Weeke B* (Eds.) A manual of quantitative immunoelectrophoresis. Methods and applications. Scand. J Immunol. *2* Suppl. 1 61–67 1973

Laskowski M Kassell B & Hagerty G A crystalline trypsin inhibitor from swine colostrum. Biochim. Biophys. Acta *24* 300–305 1957

Lowry O H Rosebrough N J Farr A L & Randall R J Protein measurements with the Folin phenol reagent. J Biol. Chem. *193* 265–275 1951

Mancini G Carbonara A. O & Heremans J F Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry *2* 235–254 1965

Weeke B General remarks on principles, equipment, reagents and procedures. In *Axelsen N H Krøll J & Weeke B* (Eds.) A manual of quantitative immunoelectrophoresis. Methods and applications. Scand. J Immunol. *2* Suppl. 1 15–35 1973 a.

Weeke B Rocket immunoelectrophoresis. In *Axelsen N H., Krøll J & Weeke B* (Eds.) A manual of quantitative immunoelectrophoresis. Methods and applications. Scand. J Immunol. *2* Suppl. 1 37–46 1973 b.

Acta path. microbiol. scand. Sect. C, 85 449–453 1977

RELATIVE RATES OF THE NON-COVALENT AND COVALENT BINDING OF SECRETORY COMPONENT TO AN IgA DIMER

E. Lindh and I. Björk

Department of Medical and Physiological Chemistry, College of Veterinary Medicine
The Swedish University of Agricultural Sciences, Uppsala, Sweden

Lindh, E. & Björk, I. Relative rates of the non-covalent and covalent binding of secretory component to an IgA dimer. Acta path. microbiol. scand. Sect. C, 85 449–453 1977

The rate of the binding of free secretory component to an IgA dimer *in vitro* was studied by high-voltage gel electrophoresis at different times after mixing. The rate of the formation of the covalent component of the interaction (i.e. the disulphide interchange reaction) was monitored separately by denaturing the proteins in 6 M guanidine hydrochloride at different times after mixing and subsequently estimating the amount of covalently bound secretory component by gel chromatography in the denaturing solvent. The rates of the two reactions could not be distinguished in experiments at 37° or 20° C. At 4° C, however, secretory component bound to the IgA dimer almost as rapidly as at higher temperatures, while the rate of the disulphide interchange was considerably lower. This indicates that the noncovalent interactions are the primary type of bonds formed between secretory component and IgA, and that the formation of these bonds initiate the disulphide interchange reaction, the rate of which is highly dependent on temperature.

Key words: IgA, secretory; immunoglobulin fragments; secretory component.

I. Björk, Department of Medical and Physiological Chemistry, College of Veterinary Medicine, The Biomedical Center, Box 575, S-751 23 Uppsala, Sweden.

Received 16   77   Accepted 16 ii.77

Secretory immunoglobulin A, the main antibody of human secretions, differs from all other immunoglobulins in that it contains a specific polypeptide chain, the secretory component, which is firmly attached to the remainder of the molecule by both non-covalent and covalent (i.e. disulphide) bonds (3, 9, 13–15). The function of secretory component remains uncertain, although most evidence suggests that it may serve as a receptor for IgA, directing the transport of the immunoglobulin through the epithelial cells of the mucosal membrane (6, 7). Free secretory component has been shown to bind to dimeric myeloma IgA *in vitro*. This reaction yields a protein complex indistinguishable from native secretory IgA and can thus be used as a model for the attachment of secretory component to IgA *in vivo* (4, 5, 10, 12). Both the non-covalent and covalent bonds present in native secretory IgA are formed during the *in vitro* binding of secretory component to IgA. It has tacitly been assumed that the non-covalent interactions are the primary bonds formed in this reaction, and that these interactions initiate a disulphide interchange leading to the formation of the

hibiting capacity in serum digestive contents, urine and organs of the developing pig. Enzymologia *43* 8–103 1972

Carlsson L. C T & *Karlsson B W* Electrophoretically separable trypsin inhibitors of the developing pig Enzyme *14* 261–274 1973

Carlsson L. C T Bergelin I S S & *Karlsson B W* Trypsin inhibition in urine of developing neonatal pigs and in sow s colostrum. Enzyme *18* 176–188 1974

Chauvet J & *Acher R.* Isolation of trypsin inhibitor (Kunitz inhibitor) from bovine ovary by affinity chromatography through trypsin sepharose. FEBS Letters *23* 317–320 1972.

Harboe N & *Ingild A* Immunization, isolation of immunoglobulins, estimation of antibody titre. In *Axelsen N H Krøll J* & *Weeke B* (Eds.) A manual of quantitative immunoelectrophoresis Methods and applications. Scand. J Immunol. *2* Suppl. *1* 161–164 1973

Krøll J Line immunoelectrophoresis. In *Axelsen N H Krøll J* & *Weeke B* (Eds.) A manual of quantitative immunoelectrophoresis. Methods and applications. Scand. J Immunol. *2* Suppl. 1 61–67 1973

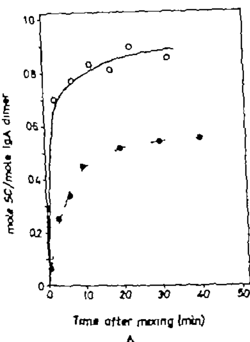
Laskowski M Kassell B & *Hagerty G* A crystalline trypsin inhibitor from swine colostrum. Biochim. Biophys. Acta *24* 300–305 1957

Lowry O H., Rosebrough N J., Farr A L. & *Randall R J* Protein measurements with the Folin phenol reagent. J Biol. Chem. *193* 265–275 1951

Mancini G Carbonara A O & *Heremans J F.* Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry *2* 235–254 1965

Weeke B General remarks on principles, equipment, reagents and procedures. In *Axelsen N H Krøll J* & *Weeke B* (Eds.) A manual of quantitative immunoelectrophoresis. Methods and applications. Scand J Immunol. *2* Suppl. 1 15–35 1973 a.

Weeke B Rocket immunoelectrophoresis. In *Axelsen N H Krøll J* & *Weeke B* (Eds.) A manual of quantitative immunoelectrophoresis. Methods and applications. Scand. J Immunol. *2* Suppl. 1 37–46 1973 b.



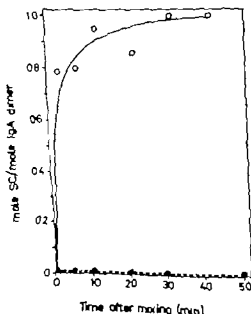


Fig 1 Number of moles of secretory component (SC) bound per mole of IgA Nor dimer by both non-covalent and covalent bonds (open circles, solid line) or by covalent bonds only (filled circles, broken line) at different times after mixing of the reactants at 37 C (A) or 4 C (B) *The final* concentration of secretory component was 3.6 μM and that of IgA dimer 1.8 μM. The *solvent* was 0 01 M sodium phosphate buffer pH 7 0. The total interaction was measured by polyacrylamide gel electrophoresis; a volume of 10 μl of the secretory component-IgA dimer mixture was applied *to* each gel. The covalent interaction was analysed by gel chromatography in 6 M guanidine hydrochloride 75 μl of the mixture was added to 225 μl of 8 M guanidine hydrochloride, and this solution was then applied to the column.

by gel chromatography in the denaturing solvent (11) and the number of moles of secretory component bound per mole of IgA was calculated *as in the gel electrophoretic* analysis. The disulphide interchange reaction was found to stop almost instantaneously on the addition of guanidine hydrochloride This procedure, therefore, does not have the disadvantages of the gel electrophoretic method and was found to give more easily interpretable results.

The rate of the reaction between secretory component and the IgA Nor dimer was first studied at 37 C (*Fig 1A*) *In this* and all subsequent experiments a molar ratio of 2 1 between secretory component and IgA dimer *was used. The total binding analysed by the* gel electrophoretic method, was found to proceed at such a rapid rate that the major part of the reaction had occurred before the reacting species could be sufficiently separat*ed from each other The covalent binding* could be followed more accurately and may have occurred somewhat more slowly although this cannot be concluded definitely due to the great uncertainty of the gel elec*trophoretic method. The curve for the total* binding was not analysed further because of this uncertainty while the covalent reaction data was plotted in the conventional manner for a second-order reaction. However this *plot was markedly curved, thus indicating* that the kinetics of the reaction are more complex than second-order

In several experiments the temperature of the reaction was decreased in an attempt to

disulphide bonds of the complex (4 5 11). The results of this investigation confirm this proposed reaction sequence.

MATERIALS AND METHODS

Dimeric myeloma IgA was obtained from the serum of a patient Nor by a method outlined in an earlier paper (2). This myeloma protein was found to bind an equimolar amount of secretory component by criteria developed in a previous investigation (10). Secretory component was purified from human milk by a method also described previously (2 10).

Labelling of proteins with [125]I was carried out by the iodine monochloride method as modified by *Freeman* (8). The gamma radiation of the nuclide was measured in a Searle Model 1195 Gamma Counter (Searle Analytic Inc., Des Plaines, Ill. U.S.A.).

Polyacrylamide gel rod electrophoresis was performed with the Pharmacia GF4 gel electrophoresis system (Pharmacia Fine Chemicals, Uppsala, Sweden). A gel concentration of 5 per cent (w/v) was used, and the buffer was 0 02 M sodium phosphate pH 7 0. The samples were layered on top of the gels after the addition of small amounts of sucrose no spacer gel was employed. The experiments were run at a potential of 40–50 V/cm for 60 min at 20 C or for 90 min at 5 C with efficient cooling of the gels by circulating buffer. After the runs the gels were sliced in 2 mm slices, and the radioactivity of each slice was counted.

Gel chromatography on CL-Sepharose 4B (Pharmacia Fine Chemicals) in 6 M guanidine hydrochloride, containing 0 05 M sodium acetate buffer pH 4.5 was carried out in 1.5 × 90 cm columns with a flow rate of 6 g of solvent per hour. The columns were calibrated with dimeric IgA and secretory component in the non reduced state.

Protein concentrations were determined spectrophotometrically using specific absorption coefficients of 12 1 for secretory component (2) and 13 0 for the IgA Nor dimer. The latter value was determined by measuring the concentration of a protein solution of known absorbance with the interference optics of the analytical ultracentrifuge (1). This procedure gives an accuracy of about ± 5 per cent.

RESULTS AND DISCUSSION

In preliminary experiments, several physicochemical techniques, viz. ultraviolet difference spectroscopy circular dichroism, fluorescence and fluorescence depolarization, were investigated as potential means of monitoring the kinetics of the interaction between secretory component and IgA dimer in solution. None of these methods was found to produce sufficiently large changes to allow any quantitation of the rate of the reaction. Consequently several rapid separation methods were also tried. The method finally adopted involved separation of the secretory component IgA dimer complex from the unreacted proteins by high voltage polyacrylamide gel rod electrophoresis at different times after mixing of free secretory component and IgA. The reaction was quantified by the use of [125]I labelled secretory component. The fraction of the total amount of secretory component which bound to IgA could be obtained by radioactivity measurement of the gels after slicing and the number of moles of secretory component bound per mole of IgA could then be calculated from the total amounts of secretory component and IgA dimer added to the reaction mixture. Although this method was the most rapid of all separation methods tried it was not satisfactory. It was used only because no better method could be found. The main drawback of the procedure was that the separation of complex from reactants required 5 to 7 min, so that the part of the reaction occurring within this time could not be studied. Moreover considerable uncertainty in the exact duration of the reaction was also introduced. To compensate for this uncertainty as far as possible, half of the time necessary for separation of the proteins (i.e. 3 min) was added to all reaction times.

The gel electrophoretic method discussed above measured the rate of the total interaction between secretory component and the IgA dimer i.e. the faster of the rates of the non-covalent and covalent interactions. The rate of the covalent reaction (i.e. the disulphide interchange) could be studied separately by rapidly denaturing the complex and the reacting proteins in 6 M guanidine hydrochloride at different times after mixing radio-labelled secretory component and IgA dimer. The fraction of secretory component covalently bound to IgA was then estimated

secretory piece. Acta path. microbiol. scand. Sect. B, 79 163–188, 1971

4 *Brandtzaeg, P* Human secretory immunoglobulins. VI. Association of free secretory piece with serum IgA *in vitro* Immunol. 29 323–332, 1971

5 *Brandtzaeg, P* Characteristics of SC-Ig complexes formed *in vitro* In *Mestecky J & Lawton A. R.* (Eds) The Immunoglobulin A System, Plenum Press, New York and London 1974, p. 87 97

6. *Brandtzaeg, P* Mucosal and glandular distribution of immunoglobulin components Differential localization of free and bound SC in secretory epithelial cells. J Immunol. *112* 1553–1559 1974

7 *Brandtzaeg, P* Mucosal and glandular distribution of immunoglobulin components Immunohistochemistry with cold ethanol-fixation technique. Immunology 26 1101–1114 1974.

8. *Freeman T* Trace labelling with radioiodine I *Weir D M* (Ed) Handbook of Experimental Immunology Blackwell, Oxford and Edinburgh, 167 p. 597–607

9 *Hurlimann J Waldesb M M & Zuber C* Human salivary immunoglobulin A. Some immunological and physicochemical characteristics. Biochim. Biophys. Acta *181* 393–403 1969

10. *Lindh E. & Björk I.*, Binding of secretory component to dimers of immunoglobulin A *in vitro* A model for the interactions of the two moieties in secretory immunoglobulin A. Eur J Biochem. *45* 261–268, 1974

11 *Lindh, E. & Björk, I.*: Binding of secretory component to dimers of immunoglobulin A *in vitro*. Mechanism of the covalent bond formation. Eur J Biochem. *62* 263–270 1976.

12. *Mach J-P* *In vitro* combination of human and bovine free secretory component with IgA of various species Nature *228* 1278–1282, 1970.

13. *Mestecky J., Kulhavy R. & Kraus, F W.*: Studies on human secretory immunoglobulin A. II Subunit structure. J Immunol. *108* 738–747 1972.

14 *Newcomb R. W N ... D & Stanworth D R.*: A structural study of human exocrine IgA globulin. J Immunol. *101*: 905–914 1968.

15. *Tomasi T B. & Bienenstock J.*, Secretory Immunoglobulins. Adv Immunol. *9* 1 96, 1968.

magnify the possible difference between the reaction rates of the total binding and the covalent binding only A temperature of 20 C gave essentially the same results as those obtained at 37° C, while new information could be derived from experiments at 4° C (Fig 1B) The rate of the total binding of secretory component to IgA at this temperature was not significantly different from that at 37° C. This may be due to a negligible temperature dependence of the reaction, but more likely merely reflects the great uncertainty of this type of analysis. In contrast, the rate of the covalent binding between secretory component and IgA was markedly affected no such binding could be detected within one hour at 4° C. However the reaction did not stop completely but only proceeded extremely slowly since a molar ratio of about 0.5 was found after 7 days at 4° C

The slow disulphide interchange at 4 C prompted further investigation of the temperature dependence of this reaction (Fig 2) The rate of the reaction was found to increase at increasing temperatures up to about 37° C, where it showed a temperature optimum. At higher temperatures the rate again decreased probably because of thermal disruption of certain structural features of one or both proteins necessary for the interchange reaction to occur

It is obvious from these results that at low temperature the gel electrophoretic method measures almost exclusively the rate of formation of the noncovalent interactions between secretory component and IgA. Thus these interactions are the primary type of bonds formed between secretory component and IgA dimer at this temperature The covalent disulphide bonds are formed more slowly by a disulphide interchange reaction (11) which is initiated by the non-covalent association of the two proteins It is reasonable to assume that the same situation also applies to interaction at physiological temperature, although the rates of both reactions (especially that of the covalent reaction) then are appreciably higher The fact that the com-

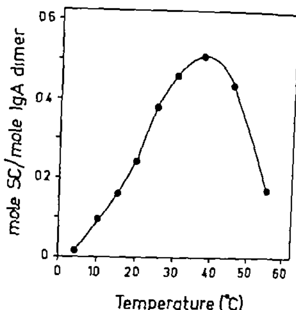


Fig 2 Number of moles of secretory component (SC) bound per mole of IgA Nor dimer by covalent bonds after a reaction time of 30 min at different temperatures. The conditions of the experiment were as in Fig 1

plex, stabilized by noncovalent bonds only is readily formed at low temperature, suggests that the disulphide bonds between secretory component and IgA do not confer any *significant stability to the complex.* Moreover the slow rate of the disulphide interchange at 4 C means that the noncovalent reaction can be studied at this temperature without the covalent reaction interfering significantly with the measurement

---

This investigation was supported by a research grant from the *Swedish Medical Research Council* (Project No 04212) We are greatly indebted to Miss A *Larsson* for excellent technical assistance

REFERENCES

1 *Babul J & Stellwagen E.* Measurement of protein concentration with interference optics Anal Biochem. *28* 216–221 1969
2. *Björk I & Lindh E* Gross conformation of human secretory immunoglobulin A and its component parts Eur J Biochem. *45* 135 145 1974
3 *Brandtzaeg, P* Human secretory immunoglobulins 3 Immunochemical and physicochemical studies of secretory IgA and free

tion of peripheral lymphocytes in 72 children who had been found previously to have low IgA levels in serum and saliva with or without a simultaneous tendency to develop atopic diseases.

PATIENTS AND METHODS

Patients. The series comprises 72 children aged 4 to 16 years who were reported to have low IgA levels associated with chronic tonsillitis (*Østergaard* 1975 1976 1977 a) or bronchial asthma (*Østergaard* 1977 b). In the present study the children were divided into three groups. 1) 24 children with a present median age of $11^7/_{12}$ years, who approximately 3½ years earlier had had their tonsils excised because of chronic and recurrent tonsillitis, originally the group comprised 28 children. 2) 28 children with a present median age of 6 / years, who on examination one year earlier had been found to has con-siderable carrier rate of pathogenic bacteria in connection with asthma often preceded by respiratory infections, originally this group comprised 32 children. 3) 20 children with a present median age of $12^1/_{12}$ years, who suffered from severe attacks of extrinsic asthma, but without a tendency to develop respiratory infections, originally this group comprised 22 children. The patients will henceforth be referred t as Group 1 Group 2, and Group 3.

Six Group 2 patients and eight Group 3 patients were treated continuously with bronchodilators, and only one of the children with asthma was treated with corticosteroids. None of the Group 1 children received drugs.

Controls. 70 healthy children from the same geographical area as the patients were selected with regard to age to match the patients. None of the controls had experienced recurrent infections or atopy and none had been subjected to adeno-tonsillectomy. The controls in the present study are not the same as those who participated in earlier studies on the same patients. The results of studies on 70 healthy controls are presented elsewhere (*Østergaard* & *Eriksen* 1977).

Preparation of lymphocyte. For isolation of the lymphocytes used in the evaluation of subpopulations of lymphocytes and Ig-containing blast cells, venous blood was drawn into an equal volume of RPMI 1640 (Biocult, Glasgow) supplemented with streptomycin (0.5 mg/ml) penicillin (500 IU/ml) heparin without preservation (15 IU/ml) and glutamin (1.5 μM). This mixture will henceforth be referred to as medium. The blood was drawn aseptically into sterile tube (110×17 mm, NUNC, Roskilde Denmark). The subsequent procedures were carried out within one hour.

Separation of lymphocytes. 6 ml of the blood medium mixture was layered on 3 ml of a Ficoll-Isopaque preparation according to the method of *Bøyum* (1967) and centrifugated at 2000 rev/ml for 20 minutes. The mononuclear cells were isolated from the interphase with a pasteur pipette and washed three times in medium. Finally the mononuclear cells were adjusted to a suspension of 5×10^6 cells per ml of medium.

The viability of the lymphocytes was tested by the Trypan blue exclusion test; this was usually more than 95 per cent. The percentage of granulocytes, evaluated on smears with toluidine blue was generally less than 1 per cent. Differential counts on peripheral lymphocytes were performed in haemocytometer. The number of lymphocytes in the patients and the controls was never less than 2000 per microlitre.

E-rosettes. A modification of the method described by *Jondal et al.* (1972) was used. Sheep red blood cells (SRBC) drawn in Alsever's solution, were obtained from Statens Seruminstitut, Copenhagen. SRBC were pretreated with papain (Merck). The papain solution used was prepared as follow: 6 g papain (= 72,000 Units) was dissolved in 600 ml PBS (pH 5.9) resulting in a final solution of 120 units of papain per ml. 0.25 ml of this solution was diluted in 2.25 ml 0.9 per cent NaCl before use. Forty drops of SRBC were transferred to the papain solution and incubated at 37 C for 30 minutes. The cells were washed four times with 0.9 per cent NaCl, and 100 microlitres of the cell pellet was transferred to a plastic tube with 8 ml medium and 2 ml human AB serum (heat inactivated and absorbed with SRBC) resulting in a final suspension of 0.5 per cent SRBC.

100 microlitres of the lymphocyte suspension was added to the SRBC solution and incubated at 37° C for 15 minutes. The mixture was spun at 500 rev/min for 15 minutes and incubated at 4 C for 18 hours. The cell pellet was resuspended gently by rolling the tubes between the palms, and the counting of T-rosettes was performed in haemocytometer. The percentages of erythrocyte rosette-forming cells (E-RFC) were evaluated under light microscope at 400 × magnification. The samples were examined in duplicate. Two hundred cells were counted, and the average of the two counts was determined. Lymphocytes with three or more surface-bound SRBC were counted as rosettes.

Immunoglobulin-(Ig-) bearing cells. The procedure used was essentially that of *Winchester* & *Fu* (1976). The mononuclear cell layer obtained by Ficoll-Isopaque centrifugation, was washed in medium at 2000 rev/min for 10 minutes. The cell pellet was adjusted t 3×10^6 cells per ml in medium, and 0.025 ml was transferred to a Vidal tube and incubated at 37 C for 30 minutes with a latex suspension (Difco, particle size 0.8 micro-

Acta path. microbiol scand. Sect. C 85 454–462, 1977

B- AND T-CELLS AND INTRACELLULAR Ig-SYNTHESIS OF PERIPHERAL LYMPHOCYTES IN CHILDREN WITH ASTHMA AND/OR PREVIOUS ADENO-TONSILLECTOMY

Poul Aabel Østergaard

Department of Clinical Chemistry Aalborg Hospital South, and
Department of Paediatrics, Blood Bank and Pathological Institute,
Aalborg Hospital North, Denmark

Østergaard, P Aa. B- and T-cells and intracellular Ig-synthesis of peripheral lymphocytes in children with asthma and/or previous adeno-tonsillectomy Acta path. microbiol. scand. Sect. C, 85 454–462 1977

Studies of IgG IgA and IgM in serum, the number of circulating T and B-cells, and the number of Ig-containing blast cells after pokeweed mitogen stimulation of peripheral lymphocytes *in vitro* were performed in children who had been found previously to have low IgA levels in serum and saliva. The first group comprises 24 children with tonsillectomy performed approximately 3½ years earlier among whom several cases of atopic diseases had been found In these children low IgA levels were found continuously while the numbers of B- and T-cells and the number of IgG IgA, IgM and IgE-containing blast cells were within normal levels. The second group comprises 28 children with recurrent and chronic respiratory infections which often preceded attacks of asthma. Very low IgA levels were found in these children associated with a decreased number of T-cells, increased numbers of lymphocytes with membrane bound IgA and IgE and an increased number of IgE-containing blast cells. The third group comprises 20 children with severe extrinsic asthma. In these children, slightly decreased levels of IgA and low levels of IgM were found. Furthermore, the number of circulating T-cells was reduced and, in addition, an increased number of lymphocytes with surface IgE was observed

Key words B- and T-cells intracellular Ig synthesis asthma adeno-tonsillectomy

P Aa. Østergaard Department of Pediatrics, Aalborg Hospital North,
DK 9100 Aalborg Denmark.

Received 30.iv 77 Accepted 28.vii 77

Increasing evidence of a possible link between atopic diseases and defective or immature development of the IgA system has accumulated (*Kaufman & Hobbs* 1970 *Taylor et al* 1973 *Buckley* 1975) Furthermore, in recent years, a connection between atopic dermatitis and a dysfunction of cell-mediated immunity has been observed (*Luckasen et al* 1974 *Andersen & Hjorth* 1975)

The aim of the present study was to investigate the number of circulating B- and T-cells and the number of Ig-containing blast cells after pokeweed mitogen stimula

TABLE 1 *Ranges and Medians of Serum Immunoglobulin Levels in the Various Groups of Patients and their Controls*

	Serum Immunoglobulin Levels IU/ml		
	IgG	IgA	IgM
Group 1			
Range	44–155	18–114	52–225
Median	90	41	64
Controls			
Range	88–275	38–255	54–300
Median	125	82	110
Group 2			
Range	75–245	0–102	44–205
Median	112	26.5	69
Controls			
Range	82–168	41–220	52–280
Median	121	74	91
Group 3			
Range	50–165	40–205	48–188
Median	106	68	72
Controls			
Range	68–170	52–180	61–225
Median	104	81	110

same children one year earlier (*Østergaard* 1977 a). In four of these children, atopic diseases were found also among their nearest relatives.

In the 28 Group 2 children, two of them no longer had a tendency to develop recurrent respiratory infections or asthma. In one, a relapse of nephrotic syndrome had developed. All the 20 Group 3 children had repeated and severe attacks of asthma.

Serum immunoglobulin levels in the patients and the controls are shown in Table I. Serum IgG and IgM levels of Group 1 patients were significantly reduced compared with their age related controls ($p < 0.01$ and < 0.02, respectively). In addition, median IgA was still low in these children (41 IU/ml) and the difference from the median IgA in their controls (82 IU/ml) was significant ($p < 0.01$). Median serum IgA of Group 2 patients was very low (26.5 IU/ml) and compared with the median IgA in their controls (74 IU/ml) the difference was significant ($p < 0.0025$). Concerning serum IgG levels, no difference between Group 2 patients and their controls was found, whereas median serum IgM of these patients (69 IU/ml) was slightly reduced compared with the median IgM of their controls (91 IU/ml) ($p < 0.05$). As regards Group 3 patients, slightly but not significantly reduced IgA levels and significantly reduced IgM levels ($p < 0.02$) were found, whereas serum IgG levels were within normal levels in these patients.

After the tonsillectomy performed in Group 1 children in 1973 a reduction of their serum immunoglobulin levels had been demonstrated (*Østergaard* 1977 a). By means of the Wilcoxon test, comparison was made between the serum immunoglobulin levels of

TABLE 2 *Ranges and Medians of Membrane-Ig on Circulating Lymphocytes and of Rosette-forming Lymphocytes (E-RFC) in Group 1 Patients and Their Controls*

	E-RFC %	Ig-bearing lymphocytes %				Polyvalent
		IgG	IgA	IgM	IgE	IgG IgA, IgM %
Patients						
Range	44–78	1–5	1–4	9–17	1–6	13–24
Median	71	2.5	1.0	12	1.5	18
Controls						
Range	61–74	0–5	0–5	8–15	0–3	11–21
Median	69	2.0	1.5	10.5	1.0	16
P	> 0.1	> 0.2	> 0.2	> 0.1	> 0.1	> 0.2

metre) in order to label contaminating monocytes with latex and to avoid capping of the Fc fragments of IgG (*Lobo et al.* 1975)

The cells were washed twice in Hanks solution at 2000 rev/min for 10 minutes and stained with 0 025 ml of a 1 10 dilution (in PBS) of FITC-conjugated rabbit anti-human IgG IgA, IgM and IgE as well as with a polyvalent FITC-conjugated rabbit-anti-human Ig specific for kappa and lambda chains of IgG IgA and IgM Incubation was made for 30 minutes at 4 C. (For details concerning the characteristics of the antisera used see below) The non-bound conjugate was removed by washing twice with Hanks' solution and the cell pellet was resuspended in the remaining droplet after removal of the supernatant. Specific staining of the conjugated antisera was blocked by exposing the lymphocyte suspension to unlabelled Ig antisera for 15 minutes before staining with the conjugated antisera. Furthermore, exposure of the lymphocytes for 15 minutes to unlabelled rabbit anti human albumin did not change specific staining with the labelled antisera

One drop of the cell pellet was placed on a microscope slide and mounted with cover slip. The wet slides were examined immediately using an oil immersion objective of 100 × in a Leitz Orthoplan fluorescence microscope with incident light to identify the various cell types. The microscope was equipped with a HBO mercury lamp, a 4 mm BG 38 glass filter one KP 490 interference filter and a dichroic mirror for selection of excitation light. Of the non-phagocytosing cells, 200 were examined, and the percentage of cells with a bright surrounding or semicircular microgranular ring of fluorescence was determined. The slides were made in duplicate, and the average of the two counts was determined.

In the evaluation of B- and T-cells, a healthy member of the hospital staff served as weekly control (5 weeks) The number of B- and T-cells in this individual was found to be very constant.

Intracellular Ig synthesis This was performed in ten Group 1 and ten Group 2 children with low IgA levels in serum. Twenty healthy children with normal immunoglobulin levels served as controls. The method used was essentially that of *Waldmann et al* (1974) and *Broom et al* (1976) 0 25 ml of a lymphocyte suspension of 1×10^6 cells per ml in medium supplemented with 10 per cent autologous serum was set up in duplicate. Stimulated cultures received 10 microlitre pokeweed mitogen (PWM MEDA, Copenhagen) diluted 1 100 in medium. Cultures from the patients without PWM and cultures from the controls in the presence or absence of PWM served as controls.

All cultures were incubated in loosely-capped, round bottomed, sterile plastic tubes for seven days at 37 C in a moistened 5 per cent CO at mosphere. On day 4 the cultures were supplemented with 0 1 ml fresh medium with serum but without PWM On the termination of the culture period, the cultures were examined with an inverted microscope to determine the percentage of proliferating blasts. After washing twice in Hanks solution, 0 025 ml of the cell pellet was incubated at room temperature for 30 minutes with 0.025 ml of the conjugated IgG IgA, IgM and IgE antisera. The procedures then carried out were essentially the same as those performed for the determination of Ig-bearing cells. The wet slides were examined in duplicate. Two hundred blast like cells (large mononuclear cells with an eccentric nucleus and sparse cytoplasma) were examined for bright fluorescence of the cytoplasma, using incident light to identify the various cell types The average of the two counts was determined.

The *rabbit-anti-human IgG IgA and IgM sera* conjugated with FITC, were purchased from Dacopatts, Copenhagen. The fluorescin/protein ratios (F/P ratios) of these antisera were 0 65 ± 0 05 The FITC-conjugated *rabbit-anti-human IgE serum* was obtained from Behringwerke AG Germany The F/P ratio of this antiserum was 2.6

Serum IgG IgA and IgM assays were performed by the Department of Clinical Chemistry Aalborg Hospital South. Rabbit anti-human IgG IgA and IgM sera, obtained from Dacopatts, Copenhagen, were used. Pooled human serum, calibrated in terms of the WHO reference preparation (*Rowe et al.* 1970) was used as a standard. The day-to-day precision of the determinations (n = 14 days) was 6 8 per cent, 5 7 per cent and 5 6 per cent for IgG IgA and IgM, respectively The serum standard was the same as that used in earlier studies with the same patients. The variation of the IgG and IgA serum standard was less than 5 per cent. However a decrease of 10 per cent of the IgM serum standard was found.

Statistical evaluation of the results was for unpaired observations performed by the Mann-Whitney test. In the case of paired observations, the Wilcoxon test was used. A significance level of 5 per cent was chosen

RESULTS

The patients have been described in greater detail previously (*Østergaard 1976 1977 b*) In the present investigation, all the 24 Group 1 children were found to be healthy except for six, who suffered from atopic diseases (three with asthma, two with atopic dermatitis, and 1 with atopic rhinitis) In one child atopic dermatitis and urticaria had developed since an investigation on the

TABLE 5. *Ranges and Medians of Serum IgA Levels and Number of Ig-containing Blast Cells after PWM-stimulation of Peripheral Lymphocytes in Ten Group 1 Patients and Their Controls*

	Serum IgA IU/ml	Ig-containing Blast Cells (%) IgG	IgA	IgM	IgE
Patients					
Range	12–56	1–4	0–4	6–13	0–3
Median	36	2.5	2.0	9.5	1.5
Controls					
Range	48–116	0–4	0–4	5–10	0–4
Median	78	3.0	1.5	8.5	1.5
P =	< 0.01	> 0.05	> 0.05	> 0.05	> 0.1

TABLE 6. *Ranges and Medians of Serum IgA Levels and Number of Ig-containing Blast Cells after PWM-stimulation of Peripheral Lymphocytes in Ten Group 2 Patients and Their Controls*

	Serum IgA IU/ml	Ig-containing Blast Cells (%) IgG	IgA	IgM	IgE
Patients					
Range	0–42	0–3	1–3	5–11	1–6
Median	24	2.5	1.0	7.0	3.0
Controls					
Range	41–122	1–3	0–3	4–11	0–4
Median	69	2.0	1.5	6.5	1.0
P =	< 0.01	> 0.1	> 0.1	> 0.1	< 0.01

within the range of that observed in the controls. As regards the results of the kappa-lambda staining of peripheral lymphocytes, there was a slight increase in these cells ($p < 0.05$).

Monocytes with ingested latex particles were found at a level of 5 to 10 per cent of the total lymphocyte population. Some of the monocytes had a fluorescing membrane, but these cells were excluded from the counts of the Ig-bearing lymphocytes.

The results of the PWM-stimulated culture in some of the Group 1 and Group 2 patients are shown in Tables 5 and 6. No difference was observed between patients and controls as regards the blast cell response in the presence of PWM ($p > 0.1$). Cultures which did not receive PWM showed only a few blast cells.

As regards the different Ig-containing blast cells, no difference was found between the patients in Group 1 (Table 5) and their controls. Conversely, in the ten Group 2 patients (Table 6) there was an increase in IgE-containing blast cells ($p < 0.01$). In spite of the increase in IgA-bearing lymphocytes observed in these patients, no difference in IgA-containing blast cells was found between the patients and the controls. The number of IgG and IgM-containing blast cells in Group 2 patients was within the range of that found in the controls. Control cultures without PWM did not show more than an occasional Ig-containing cell.

TABLE 3 *Ranges and Medians of Membrane Ig on Circulating Lymphocytes and of Rosette-forming Lymphocytes (E RFC) in Group 2 Patients and Their Controls*

	E-RFC %	Ig bearing lymphocytes % IgG	IgA	IgM	IgE	Polyvalent IgG IgA, IgM %
Patients						
Range	42–81	0–6	0–6	10–17	0–7	14–26
Medians	53	2.0	3.0	11.5	19.5	19.5
Controls						
Range	55–82	0–5	0–4	8–16	0–3	8–20
Medians	70	2.0	1.0	10	1.0	14.5
P =	< 0.001	> 0.1	< 0.001	> 0 [illegible]	< 0.001	< 0.025

TABLE 4 *Ranges and Medians of Membrane-Igon Circulating Lymphocytes and of Rosette-forming Lymphocytes (ERFC) in Group 3 Patients and Their Controls*

	E-RFC %	Ig bearing lymphocytes % IgG	IgA	IgM	IgE	Polyvalent IgG IgA, IgM %
Patients						
Range	41–75	1.6	1–4	5–11	2–6	11–25
Median	57	2.5	1.5	9.5	3.5	17
Controls						
Range	61–80	0–5	0–4	4–14	0–4	8–21
Median	73.5	2.0	1.0	10	1.5	14.5
P =	< 0.01	> 0.1	> 0.05	> 0.1	< 0.01	< 0.05

these patients in 1976 and the serum immunoglobulin levels in the present investigation (1977) The study revealed that a decrease in serum IgA ($p < 0.01$) as well as in serum IgG ($p < 0.01$) and serum IgM ($p < 0.02$) had occurred.

The results of the lymphocyte subpopulation studies are shown in Tables 2 3 and 4 In Group 1 children (Table 2) no differences in the percentages of E-rosette forming cells *(E-RFC) or in IgG IgA IgM and* IgE-bearing cells between the patients and the controls were found. However three of the patients with atopic diseases had rather low percentages of E-RFC (44 48 and 51 *per cent, respectively)* and in addition in two of these three children high numbers of peripheral *lymphocytes with membrane* bound IgE were found (5 and 6 per cent, respectively)

In Group 2 patients (Table 3) a significant decrease in E-RFC ($p < 0.001$) and a significant increase in both IgA and IgE-bearing lymphocytes was found ($p < 0.001$) compared with the age-related controls. Furthermore a significant increase was observed in the total number of Ig-bearing Cells, as determined by the specific kappa lambda serum ($p < 0.0025$) This was presumably due to the increase in IgA and IgE-bearing lymphocytes in these patients.

In Group 3 patients (Table 4) a reduced number of E-RFC was found ($p < 0.01$) In addition the number of IgE-bearing cells was increased ($p < 0.01$) whereas the number of IgG- IgA and IgM bearing cells was

per cent failed to mount a delayed hypersensitivity reaction. These patients had very high serum IgE levels and, in addition, they all had elevated serum IgA levels. Conversely in a study of 69 patients with asthma Saha et al. (1975) found that 16 per cent were deficient in IgA and furthermore had the highest levels of serum IgE found in these patients.

In the younger patients with asthma in the present study the increased number of IgA-bearing lymphocytes may be due to a compensatory increase caused by a negative feed-back mechanism associated with the low IgA levels observed in these patients. However, normal numbers of IgA-containing blast cells were invariably found, but, as secretion studies have not been performed, the possibility exists that intracellular IgA was not secreted in normal amounts. This may be due to a regulatory intrinsic B-cell defect or to a reduced T-cell helper function, the latter possibility in view of the decreased number of E-RFC observed in these patients. Furthermore in both the younger and the older patients with asthma, the low number of E-RFC found in connection with the increased number of IgE-bearing cells might perhaps be due to a deficient T-cell suppressor function, in view of the observation that T-cells exert an inhibitory effect on the IgE antibody formation in rats (*Tada et al* 1975).

However in the previously tonsillectomized children with low IgA levels, normal subpopulations of both lymphocytes and Ig containing blast cells were found, except for a few patients with atopic diseases. These findings emphasize that low IgA levels are not always associated with a demonstrable thymic dysfunction.

The study was supported by *the Danish Medical Research Council* (Grant no. 512 7178) and by *Det Lægevidenskabelige Forskningsfond for Nordjyllands Amt*. The author is greatly indebted to *Dr Grethe Sønderstrup* the Tissue Typing Laboratory University Hospital, Copenhagen (Rigshospitalet) for valuable technical advice.

REFERENCES

Ammann A. J., Cain, W. A., Ishizaki K. Hong R. & Good R. A. Immunoglobulin E deficiency in ataxia-telangiectasia. New Engl. J Med. *281* 469–472, 1969

Ammann, A. J., Meuwissen H. J. Good R. A. & Hong, R. Successful bone marrow transplantation in a patient with humoral and cellular immunity deficiency. Clin. exp. Immunol. *7* 343–353 1970.

Andersen E. & Hjorth N. B-lymphocytes, T lymphocytes and phytohemagglutinin responsiveness in atopic dermatitis. Acta Dermato-Venerol *55* 345–349 1975.

Brown, B. C., De La Concha E. G. Webster A. D. B., Janossy, G. J. & Asherson G. L. Intracellular immunoglobulin production in vitro by lymphocytes from patients with hypogammaglobulinemia and their effect on normal lymphocytes. Clin. exp. Immunol. *23* 73–77 1976

Buckley R. H., Wray B. B. & Belmaker E. Z. Extreme hyperimmunoglobulinemia E and undue susceptibility to infection. Pediatrics *49* 59–70 1972.

Buckley R. H. Clinical and immunological features of selective IgA deficiency. Birth Defects Orig. Art. Ser *11* 134 142 1975

Bøyum A. Isolation of mononuclear cells and granulocytes from human blood. Scand. J Clin Lab. Invest. *21* 77–89 1967

Church J. A., Frenkel L. D. Wright D. G. & Bellanti J. A. T lymphocyte dysfunction, hyperimmunoglobulinemia E, recurrent bacterial infections, and defective neutrophil chemotaxis in negro child. J Pediat. *88* 982–985 1976

Clough J. D. Mims, L. H. & Strober W. Deficient IgA antibody response to arsanilic acid bovine serum albumin (BSA) in neonatally thymectomized rabbits. J Immunol. *106* 1019 1025 1971

Delespesse G. Gausset P. H. Cauchie C. H. & Govaerts A. Cellular aspects of selective IgA deficiency. Clin. exp. Immunol. *24* 273–279 1976

Grove D. I. Burston T. O. Wellby M. L. Munford R. & Forbes, I. J. Humoral and cellular immunity in asthma. J Allerg. Clin. Immunol. *55* 152–163 1975

Jondal M. Holm G. & Wigzell, H. Surface markers on human T and B lymphocytes J exp Med. *136* 207–215 1972.

Kaufman H. S. & Hobbs J. R. Immunoglobulin deficiencies in an atopic population. Lancet *II* 1061–1063, 1970

Kikkawa Y. Kamimura, K., Hamajima K., Sekiguchi T. Kawai, T. Takenaka M. & Tada, T. Thymic alymphoplasia with hyper-IgE-globulinemia. Pediatrics *51* 690–696 1973

DISCUSSION

In the present study the younger children with asthma and recurrent respiratory infections had very low serum IgA levels, and, in addition, low levels of serum IgM. Furthermore in these patients, a low number of circulating T-cells an increased number of IgA and IgE-bearing lymphocytes, and an increased number of IgE-containing blast cells after PWM-stimulation of peripheral lymphocytes were observed. In the older children with asthma, slightly but not significantly reduced levels of serum IgA and significantly reduced levels of serum IgM were found in connection with a decreased number of T cells and an increased number of IgE-bearing lymphocytes. Conversely in the previously tonsillectomized children with low IgA levels in serum both the subpopulations of lymphocytes and the number of Ig bearing and Ig containing cells were normal except in a few atopic children with decreased levels of T cells and increased levels of IgE-bearing cells. On repeated occasions most of the previously tonsillectomized children had been found to have low IgE levels in serum (*Østergaard* 1976 1977 a) whereas serum IgE levels had been found to be very high in the two groups of children with asthma (*Østergaard* 1977b)

Neonatally thymectomized animals often develop IgA deficiency (*Clough et al* 1971) and patients with ataxia telangiectasia exhibit both a high frequency of selective IgA deficiency and deficient cell mediated immunity (*Ammann et al.* 1969) It is therefore tempting to consider the possibility that the differentiation abnormality in patients with IgA deficiency may be due to thymic dysfunction. However *in vivo* cellular immune responses have been normal in the majority of such patients except those with ataxia telangiectasia (*Buckley* 1975) So far the only evidence to support a T-cell deficiency in patients with low IgA levels derives from rosette formation studies.

The use of spontaneous rosette formation as a potential means of identifying human T-cells has indicated that more than 90 per cent of human thymocytes can form such rosettes and that anti-immunoglobulin reagents do not inhibit rosette formation (*Silveira et al* 1972) In addition some investigators (*Wybran & Fudenberg* 1975) have found a close correlation between E-RFC and various T-cell function tests.

The rôle of the thymus in IgA and IgE responses is emphasized by different clinical observations in patients with disturbances in IgA and IgE synthesis. *Ammann et al.* (1970) and *Schlegel et al* (1970) reported several cases of thymic dysplasia associated with IgA deficiency Furthermore, hyperimmunglobulinaemia E and an undue susceptibility to infections in connection with thymic alymphoplasia (*Kikkawa et al* 1973) or impaired cell mediated immunity (*Buckley et al.* 1972, *Church et al* 1976) have been observed. In most of these patients, however the IgA levels were found to be normal or even elevated. In a study of 75 patients with IgA deficiency among them many atopics (*Buckley* (1975) noted a reduced number of E-RFC and a normal or increased number of IgA-bearing lymphocytes in some patients. Conversely *Delespesse et al* (1976) found a normal number of IgA-bearing lymphocytes in five patients with undetectable IgA in serum but without atopic diseases. These observations and the fact that some patients with IgA deficiency later begin to synthetize normal amounts of IgA spontaneously (*Buckley 1975 Østergaard (in press)*) is in support of a regulatory abnormality affecting the differentiation of IgA bearing lymphocytes in these patients.

A reduced number of E-RFC has been found, particularly in patients with atopic dermatitis (*Luckasen et al* 1974 *Andersen & Hjorth* 1975) Furthermore, a reduced number of T-cells in connection with elevated levels of IgE bearing lymphocytes and serum IgE was noted in patients with atopic dermatitis (*Schoeph & Boehringer* 1974) and in patients with asthma and atopic dermatitis (*Strannegård et al.* 1976) In a study of 91 patients with various atopic diseases (*Grove et al* 1975) reported that almost 10

Acta path. microbiol. scand. Sect. C, 85 463-468, 1977

IMMUNOGLOBULIN CLASS OF ANTILYMPHOCYTE ANTIBODIES IN SYSTEMIC LUPUS ERYTHEMATOSUS PATIENTS AND THEIR FAMILIES

ROBERT P SEARLES, GUNNAR HUSBY and RONALD P MESSNER

Department of Medicine, Division of Rheumatology
University of New Mexico School of Medicine,
Albuquerque New Mexico 87131 U.S.A

Searles, R. P Husby G & Messner R. P Immunoglobulin class of antilymphocyte antibodies in systemic lupus erythematosus patients and their families. Acta path. microbiol. scand. Sect. C, 85 463-468, 1977

Antilymphocyte antibody (ALA) in systemic lupus erythematosus (SLE) was studied using an indirect immunofluorescent technique with air-dried, acetone-fixed lymphocytes Ninety four per cent of patients with SLE and 65 per cent of their relatives had ALA. The ALA response in SLE patients involved antibodies of all classes. However the ALA in their relatives is primarily of the IgM class Restriction of the class of ALA in asymptomatic relatives is analogous to that seen with anti-RNA antibodies and suggests that the switch to production of autoantibodies of the IgG class may be important in the pathogenesis of SLE.

Key words. Systemic lupus erythematosus antilymphocyte antibodies Ig class

Gunnar Husby Department of Rheumatology Institute of Clinical Medicine, University of Tromsø, Tromsø Norway

Received 12 iv 77 Accepted 20 viii.77

Cold reactive lymphocytotoxic antibodies have been demonstrated in a variety of conditions. Among these are autoimmune diseases such as systemic lupus erythematosus (SLE) (13 25) post-infectious states (14) and pregnancy (15) The basic methods used to detect antilymphocyte antibodies (ALA) are microcytotoxicity (24) and indirect immunofluorescence with lymphocyte suspensions (12 28) Both of these procedures favor IgM cold reactive antibodies and require viable lymphocytes. We have recently described a new method for antilymphocyte antibody detection with airdried, acetone-fixed lymphocytes as targets (8) We now present our initial data using this method to determine the class of antilymphocyte antibodies in patients with SLE and their asymptomatic family members.

Presented in part at the American Rheumatism Association Meeting June 1976, Chicago, Illinois.

MATERIALS AND METHODS

Frozen sera from 20 different SLE patients and 31 relatives were selected for study All SLE patients met the American Rheumatism Association criteria for the diagnosis of SLE (3) Their activity varied from acute untreated to stable patients controlled on prednisone and/or azathioprine. The fam-

Lobo P I Vesterveit F B & Horwitz D A Ideentification of two populations of immunoglobulin-bearing lymphocytes in man. J Immunol *114* 116–119 1975

Luckasen J R Sabad A Goltz R. W & Kersey J H T and B-lymphocytes in atopic ekzema Arch. Derm. *110* 375–377 1974

Rowe D S Anderson S G & Grab B A research standard for human serum immunoglobulins IgG IgA and IgM Bull. Wld. Hlth. Org *42* 535–538 1970

Saha K Kulpati D D & Padmini R. A study of serum Immunoglobulins including IgE and reaginic antibodies in patients with bronchial asthma in relation to the spectrum of the disease Clin. Allergy *5* 339–349 1975

Schlegel, R J Bernier G M Bellanti J A Maybee D A Osborne G B Steward J L. Pearlman D S Oulette J & Biehusen F C Severe candidiasis associated with thymic dysplasia, IgA deficiency and plasma anti-lymphocyte effects. Pediatrics *45* 926–936 1970

Schoeph E & Boehringer D IgE aand cell mediated Immunity in atopic dermatitis. J Derm. *1* 133–144 1974

Silverra N P A Mendes N F & Tolnai M E A Tissue localization of two populations of human lymphocytes distinguished by membrane receptors J Immunol. *108* 1456–1464 1972.

Strannegård I L. Lindholm L. & Strannegaard Ö T lymphocytes in atopic children. Int. Arch. Allergy *50* 684–692, 1976.

Tada T Okumura, K & Taniguchi M Cellular basis of the IgE antibody formation in the rat. In *Ishizaka K & Dayton D H* (Eds.) The biological role of the immunoglobulin E system. National Institute of Child Health and Human Development Betheada, Maryland, U.S.A., *1973 pp 89–102.*

Taylor B Norman A Orgel H., Stokes, C., Turner M & Soothill, J F Transient IgA deficiency and pathogenesis of infantile atopy Lancet *II* 111–114 1973

Waldmann T A Broder S., Blaese R M Durm M Blackman M & Strober W Role of suppressor T-cells in pathogenesis of common variable hypogammaglobulinemia. Lancet *II* 609–613 1974

Winchester R J & Fu S M Lymphocyte surface membrane immunoglobulins. Scand. J Immunol. *5* suppl. 5 77–89 1976

Wybran J & Fudenberg H H Human thymus rosette forming cells and immunologic diseases. Birth Defects. Orig Art Ser *11* 79–84 1975.

Østergaard P Aa. Presence of immunoglobulin-containing plasma cells in tonsils from tonsillectomized children Acta path. microbiol. scand. Sect. C, *83* 406–412 1975

Østergaard P Aa. IgA levels and carrier rate of *Haemophilus influenzae* and beta-haemolytic streptococci in children undergoing tonsillectomy Acta path microbiol. scand. Sect. C, *84* 290–298 1976

Østergaard P Aa. IgA levels and carrier rate of pathogenic bacteria in 27 children previously tonsillectomized. Acta path. microbiol. scand. Sect. C, *85* 178–186, 1977 a.

Østergaard P Aa. IgA levels, bacterial carrier rate and the development of bronchial asthma in children. Acta path. microbiol. scand. Sect. C, *85* 187–195 1977 b.

Østergaard P Aa. Clinical aspects of transient immunoglobulin deficiency in children (In press) Dan. Med. Bull

Østergaard P Aa. & Eriksen J B- and T-cells and Ig-containing blast cells in healthy children Acta path. microbiol. scand. Sect. C, *85* 395–397 1977

Acta path. microbiol. scand. Sect. C, 85: 463–468, 1977

IMMUNOGLOBULIN CLASS OF ANTILYMPHOCYTE ANTIBODIES IN SYSTEMIC LUPUS ERYTHEMATOSUS PATIENTS AND THEIR FAMILIES

ROBERT P. SEARLES, GUNNAR HUSBY and RONALD P. MESSNER

Department of Medicine, Division of Rheumatology
University of New Mexico School of Medicine
Albuquerque, New Mexico, 87131 U.S.A.

Searles, R. P., Husby, G. & Messner, R. P. Immunoglobulin class of antilymphocyte antibodies in systemic lupus erythematosus patients and their families. Acta path. microbiol. scand. Sect. C, 85: 463–468, 1977.

Antilymphocyte antibody (ALA) in systemic lupus erythematosus (SLE) was studied using an indirect immunofluorescent technique with air-dried, acetone-fixed lymphocytes. Ninety-four per cent of patients with SLE and 65 per cent of their relatives had ALA. The ALA response in SLE patients involved antibodies of all classes. However, the ALA in their relatives was primarily of the IgM class. Restriction of the class of ALA in asymptomatic relatives is analogous to that seen with anti-RNA antibodies and suggests that the switch to production of autoantibodies of the IgG class may be important in the pathogenesis of SLE.

Key words: Systemic lupus erythematosus; antilymphocyte antibodies; Ig class.

Gunnar Husby, Department of Rheumatology, Institute of Clinical Medicine, University of Tromsø, Tromsø, Norway.

Received 12.iv.77. Accepted 20.vii.77

Cold reactive lymphocytotoxic antibodies have been demonstrated in a variety of conditions. Among these are autoimmune diseases such as systemic lupus erythematosus (SLE) (13, 23), post-infectious states (14) and pregnancy (15). The basic methods used to detect antilymphocyte antibodies (ALA) are microcytotoxicity (24) and indirect immunofluorescence with lymphocyte suspensions (12, 28). Both of these procedures favor IgM cold reactive antibodies and require viable lymphocytes. We have recently described a new method for antilymphocyte antibody detection with airdried, acetone fixed lymphocytes as targets (8). We now present our initial data using this method to determine the class of antilymphocyte antibodies in patients with SLE and their asymptomatic family members.

Presented in part at the *American Rheumatism Association Meeting*, June, 1976, Chicago, Illinois.

MATERIALS AND METHODS

Frozen sera from 20 different SLE patients and 31 relatives were selected for study. All SLE patients met the American Rheumatism Association criteria for the diagnosis of SLE (3). Their activity ar[illegible] led from acute untreated to stable patients controlled on prednisone and/or imuran. The fam-

ilies of seven SLE probands were randomly selected for study. These families contained 31 members, divided into 21 consanguineous and 10 nonconsanguineous relatives of the respective probands. All relatives studied were close household contacts of the seven SLE patients.

The assay for ALA has been previously reported in detail (8). Briefly, peripheral blood lymphocytes were isolated from normal volunteers of varying HLA phenotypes by Ficoll Hypaque gradient centrifugation. One drop of lymphocyte suspension (4×10^6 cells/cc) was air-dried on a clean glass slide and subsequently fixed in acetone at —20 C for 5 minutes. Experiments comparing acetone fixation of lymphocytes at —30 C for 10 minutes as in our original report and —20 C for 5 minutes revealed no differences between these two methods. The preparation was then reacted in an indirect immunofluorescent technique using test serum at 1:40 and fluorescein isothiocyanate (FITC) labelled pepsin-digested rabbit anti-human Fab (anti-Fab). This reagent was chosen to eliminate any Fc binding by the staining reagent itself. The slides were then mounted in 50 per cent glycerol (pH 7.4) to be read for immunofluorescence.

In our initial report we noted approximately 35 per cent of normal sera and 100 per cent of SLE reacted with 50 per cent or more of the cells when tested undiluted against a single donor. A better distinction between normal and SLE sera was obtained at a serum dilution of 1:40. As experience was gained with this technique, reactions of ALA were noted to vary with individual donor lymphocytes. This problem was examined using 15 SLE sera and 15 normal sera against lymphocytes from 10 normal donors. Analysis of approximately 300 test results using a binominal distribution revealed that false negatives and positives were below 1 per cent if positivity was considered when a serum reacted with three or more of 10 donors and below 2 per cent if a reaction occurred with at least two of five donors. Thus, a serum was considered positive if it reacts with 50 per cent or greater test cells from two or more of five donors at a dilution of 1:40.

Additional studies were conducted using antisera monospecific for IgM, IgG, IgA, IgD and IgE antibodies. These antisera were obtained from Hyland (anti IgM, IgG) and Meloy (anti IgA, IgD, IgE) Laboratories. All were tested for specificity using Ouchterlony analysis or immunoelectrophoresis in agar. Anti-IgM and anti IgG FITC conjugates were found to be of equal strength when serial dilutions were tested against purified IgM and IgG myeloma proteins by immunodiffusion. Specificity of the anti IgE was further documented by direct immunofluorescence with IgE myeloma cells (kindly supplied by K. Nilsson, Uppsala, Sweden). Further testing using these monospecific antisera revealed pepsin digestion was not necessary to eliminate interference by Fc binding. Direct staining of the lymphocyte preparations with these monospecific sera yielded from 2 per cent to 8 per cent positive cells. These background positives were well below the 50 per cent level previously established as criteria for a positive preparation.

Lymphocytotoxicity utilized the standard microcytotoxicity method of Terasaki and McClelland (24). A serum was considered positive if it killed 20 per cent or more of the cells from at least half the panel of 10 normal donors. All sera were tested for antinuclear antibodies (ANA) using mouse liver as substrate and the same FITC labelled pepsin-digested anti-Fab as the direct staining reagent. Routine screening dilutions of sera was 1:10.

RESULTS

A comparison was made between SLE patients and their family members with respect to the incidence and class of ALA. Parallel studies of antinuclear antibodies (ANA) were also performed. Ninety percent of SLE patients and only 3 percent of relatives were positive for ANA (Table 1). Antilymphocyte antibodies were found in 94 percent of SLE patients and 65 percent of their relatives ($P = .02$). Positive ALA were found in 76 percent of the consanguineous and 40 percent of the non-consanguineous relatives that were tested. Only 5 percent of these family members with ALA had a positive test for ANA. The incidence of ALA in both patients and their relatives was significantly different from that found in normal controls ($P < .01$). The gammaglobulin level was determined in the serum of 23 of the SLE relatives, 15 of whom were positive for ALA. No elevated levels were detected.

TABLE 1. *Presence of Antinuclear Antibodies (ANA) and Antilymphocyte Antibodies (ALA) in Serum from SLE Patients and Their Family Members*

	SLE		Family	
	Number	% Positive	Number	% Positive
ANA	20	90	30	3
ALA	18	94	31	65

TABLE 2. *Occurrence of Immunoglobulin Class of Antilymphocyte Antibodies in Serum from SLE Patients and Their Family Members*

Antibody	SLE No. Positive/ No. Tested	Family No. Positive/ No. Tested
A-Fab	17/18	20/31
IgM	15/17*	10/20*
IgG	16/17	1/20
IgA	9/17	1/20
IgD†	7/17	1/20
IgE†	1/15‡	0/15‡

* Only sera positive with anti-Fab were tested for Ig class specificity.
† Positive results at dilution of ≥ 1:5.
‡ Only 15 SLE and FM sera tested due to lack of serum.

In an effort to further define these ALA, positive sera were tested against anti-sera monospecific for IgM, IgG, IgA, IgD and IgE. The sera tested were those which previously had shown a positive reaction with anti-Fab. Sera were tested at a dilution of 1:40 with anti-IgM, IgG and IgA as with the anti-Fab. Sera tested with anti-IgD were diluted to 1:5 in view of the relative low concentration of these antibodies. The results indicate that SLE patients have ALA of all the different immunoglobulin classes (Table 2). IgM and IgG ALA were found in over 80 percent of the patients. Antilymphocyte antibodies of the IgA and IgD classes were present in approximately half the patients. IgE ALA was the least common and was found in only one of the 15 patients tested. This IgE positive serum also contained ALA of the IgM and IgG classes. In contrast, relatives of SLE patients have a much more restricted pattern of ALA response. IgM ALA was found in 50 percent of sera tested while 5 percent or less had ALA of the IgG, IgA, IgD or IgE classes. There were three basic patterns of fluorescent staining noted in the studies. The rim pattern which had staining confined to the peripheral margin of the cell was associated with IgM ALA. The reticular pattern consisted of a network of fine threads and granules over the entire cell surface. This and the diffuse staining of the cells were associated with IgG, IgA, IgD and IgE ALA. Combined phase contrast and immunofluorescence microscopy showed the staining to be located on the cell surface and not the intracellular or nuclear areas.

In our initial report (8) no significant correlation was found between the lymphocytotoxicity and the acetone fixation technique for determination of ALA in SLE patients. Variability in the reaction of sera from less active SLE patients with lymphocytes from different normal donors accounted for this finding. Since SLE family members also have lesser amounts of ALA by either method, a study was done on 24 relatives to recompare the two methods using different subjects. Again no significant correlation was noted (Table 3). Further comparison of test-to-test on individual donors also failed to reveal any significant correlation.

TABLE 3. *Antilymphocyte Antibodies in SLE Family Members. Acetone-fixed Slide Versus Microcytotoxicity*

		Acetone-fixed Positive	Acetone-fixed Negative
Cytotoxicity	Positive	9	2
	Negative	9	4

P = .056

DISCUSSION

The results reported here indicate that SLE patients have ALA of all immunoglobulin classes while their relatives produce predominantly ALA of the IgM class. The 94 percent incidence of IgG ALA in the patients is significantly greater than the 5 percent in their relatives ($P < .005$). These data are similar to those reported by Talal et al. (22, 23) who demonstrated 7S IgG antibodies to RNA and DNA in SLE patients while their relatives produced predominantly 19S IgM antibodies to RNA. The switch to production of IgG antibodies in those individuals with disease is probably controlled by T

lymphocytes (1 4) Whether the production of IgG antibodies is causally related to the development of disease or represents a marker of more severe involvement is unknown Along this line, other reports (17 19) have demonstrated IgG antibodies to DNA in SLE patients with active nephritis while IgM anti DNA antibodies predominated in SLE patients without nephritis. Further investigation of the control of the IgM to IgG switch and the relative pathogenecity of IgM vs IgG antibodies in SLE appears warranted.

Antilymphocyte antibodies of the IgA IgD and IgE classes were also found in SLE sera. To our knowledge this is the first description of these classes of ALA. Because of their low concentration, IgD and IgE ALA were reported as positive if reactions occurred at titers of 1 5 or greater Through the increased sensitivity of the acetone fixation technique, the detection of these smaller amounts of ALA can now be appreciated Worth noting is that the sum of family member sera positive with class specific antibodies (12 sera) did not equal the number positive with anti Fab (20 sera) A possible explanation for this difference may be that the concentration of the individual classes of ALA were too low to be detected by the class specific antisera. *Alternatively the* $F(ab)_2$ part of the antibodies may be more easily detectable than the Fc fragment.

Binding of immune complexes to Fc receptors on lymphocyte surfaces is another possible source of error As previously reported (8) the pepsin $F(ab)_2$ portion of isolated IgG from SLE sera showed reactivity similar to that of the corresponding whole sera This demonstrates that IIF staining was due to a true antigen antibody reaction and not to binding of the Fc portion of the antibody molecule Also the FITC-conjugate rabbit anti Ig antibodies were pepsin-digested which excludes the possibility of Fc binding of the fluoresceinated antibodies.

Since sera from most family members who were positive for ALA in the test with acetone-fixed lymphocytes were negative for ANA, it is extremely unlikely *that their re*sults are complicated by reactions with the lymphocyte nuclei. This conclusion is also supported by datta showing that individual SLE sera with ANA and ALA activity do not react with acetone fixed lymphocytes from all normal donors This donor specificity is not *characteristic of ANA* but is typical of ALA as defined previously in the microcytotoxicity system (25) Results of absorption studies previously reported (8) suggestetd that nuclear antigens are not involved in the ALA reactivity using indirect immunofluorescence on lymphocytes fixed in acetone at — 30° C. Furthermore, morphologic evidence of ALA specificity was obtained using combined phase contrast and *immunofluorescent microscopy* Although somewhat indirect, the evidence of ALA specificity for lymphocyte surface antigens appears convincing. However the final proof of such specificity will require experiments with completely purified nuclear and surface antigens obtained from lymphocytes. Such reagents are not available at the present time

Both environmental and genetic factors have been implicated in the pathogenesis of SLE (5 6 10 11 20 21 30) Previous studies in our laboratory have demonstrated the presence of lymphocytotoxic antibodies in *82 percent of SLE probands* and 57 percent of their relatives (5) This phenomenon has recently been confirmed by *Malave et al* (10) The incidence of lymphocytotoxic antibody was especially notable in close household contacts (5) Anti-double-stranded RNA antibodies are also influenced by proband contact and consanguinity (6) Our experiments on ALA using acetone fixed lymphocytes are concordant with these previous data on overall antibody incidence in SLE families. The 70 per cent incidence of ALA in consanguineous and 40 percent in nonconsanguineous close household conttacts noted in the current study is in agreement with data obtained with the cytotoxicity method. These studies all suggest that environmental factors are important in the generation of ALA

Comparison of the lymphocytotoxicity and the *test with acetone-fixed* lymphocytes for

ALA determination using target lymphocytes from the same normal donors failed to show significant correlation between the two techniques. It is probable that the acetone fixation technique measures different antilymphocytic antibodies that the cytotoxicity test. The acetone fixation technique has certain technical advantages in that it can be done with a smaller donor panel, and is easily adaptable to the study of antibody classes through the use of monospecific indicator antisera. On the other hand, it has yet to be demonstrated that the test with acetone-fixed lymphocytes will detect ALA that correlate with disease activity as well as ALA detected by cytotoxicity (2, 29)

The present data clearly demonstrate that in SLE ALA are heterogeneous in antibody class. It is probable that they are also heterogeneous with respect to specificity for different antigens on the cell surface (9 16 18) and include antibodies which effect various parameters of *in vitro* lymphocyte function (7 26 27). Sorting out the relative importance of these antibodies in the pathogenesis of SLE will be a complex but hopefully illuminating task.

Supported by *Career Development Award* grant # 5KO4 AM70301-04 grant # 2 RO1 AM13780-06 from *the National Institute of Health* and Grants from the *National and New Mexico Arthritis Foundation*

REFERENCES

1. *Baker P J Stashak D F Amsbaugh D F & Prescott B* Regulation of the antibody response to type III pneumococcal polysaccharide II Mode of action of thymic-derived suppressor cells J Immunol. *112* 404–409 1974
2. *Butler W T Sharp J T Rossen, R D Lidsky M D Mittal K K & Gard D D* Relationship of the clinical course of systemic lupus erythematosus to the presence of circulating lymphocytotoxic antibodies. Arthritis Rheum *13* 231 238, 1972.
3. *Cohen A & Canoso J J* Criteria for the classification of systemic lupus erythematosus status Arthritis Rheum *15* 540–543 1972.
4. *Dause J M & Paul, W E.* Role of T lymphocytes in the humoral immune response. I Proliferation of B lymphocytes in thymus-deprived mice. J Immunol. *113* 1438–1439 1974
5. *DeHoratius, R J & Messner R. P* Lymphocytotoxic antibodies in family members of patients with systemic lupus erythematosus. J Clin. Invest. *55* 1254–1258, 1975.
6. *DeHoratius R J., Pillarisetty R Messner R. P & Talal N.* Anti-nucleic acid antibodies in systemic lupus erythematosus patients and families. J Clin. Invest. *56* 1149–1154 1975
7. *Diaz Jouanen E., Bankhurst A D & Williams, R. C., J .* Antibody-mediated lymphocytotoxicity in rheumatoid arthritis and systemic lupus erythematosus. Arthritis Rheum. *19* 133–141 1976.
8. *Husby G & Messner R. P.:* Detection of antilymphocyte antibodies in patients with systemic lupus erythematosus by indirect immunofluorescence on acetone-fixed lymphocytes. J Lab. Clin. Med. *89* 240–249 1977
9. *Lewis R. M., Borunsberg, W., Smith C & Schwartz R. S.* C-type viruses in systemic lupus erythematosus. Nature *252* 78–79 1974
10. *Malex I., P pa, R & Layrisse Z..* Lymphocytotoxic antibodies in SLE patients and their relatives. Arthritis Rheum. *19* 700–704 1976
11. *Messner R. P..* Clinical aspects of T and B lymphocytes in rheumatic diseases. Arthritis Rheum. *17* 339–346 1974
12. *Messner R. P Kennedy M S & Jelinek J G..* Antilymphocyte antibodies in systemic lupus erythematosus effect on lymphocyte surface characteristics. Arthritis Rheum. *15* 540–543
13. *Mittal, K K Rossen R. D Sharp J T., Lidsky M D & Butler W T* Lymphocytotoxic antibodies in systemic lupus erythematosus Nature *225* 1255–1256, 1970.
14. *Mottironi, V D & Terasaki P I* Lymphocytotoxins in disease. I. Infectious mononucleosis, rubella and measles. *In* Histocompatability Testing (ed. P I. Terasaki) p 301 308. The Williams & Wilkins Company Baltimore, 1970
15. *Nab S., Mickey M R., Hirata, A et al.,* Antilymphocytotoxins following immunization by pregnancy transplantation and disease. Tissue Antigens *1* 219–228, 1971
16. *Nies, K M Brown, J., Dubois, E., Quismorio F Friou G J & Terasaki P* Histocompatibility (HL-A) antigens and lymphocytotoxic antibodies in systemic lupus erythematosus (SLE) Arthritis Rheum. *17* 397–402, 1974
17. *Pennebaker J Gilliam J & Ziff M.,* (Abstract) Significance of anti-nDNA classes in serum and skin in prognosis of systemic lupus

erythematosus. Arthritis Rheum. *19* 815 1976.

18 *Persselin J Messner R. P Troup G & DeHoratius R J* (Abstract) Familial clustering of lymphocyte antigens to which systemic lupus erythematosus (SLE) lymphocytotoxic antibodies (LCA) are directed Clin. Res. *24* 134A, 1975

19 *Rothfield N & Stollar B* The relation of immunoglobulin class, pattern of antinuclear antibody and complement fixing antibodies to DNA in sera from patients with systemic lupus erythematosus. J Clin. Invest. *46* 1785–1794 1967

20 *Solheim B G & Larsen R A* Family studies in systemic lupus erythematosus. IV Presence of antinuclear factors in the total population of relatives and spouses, and the correlation to rheumatic disease. Acta Med Scand Suppl. *543* 43–53 1972

21 *Talal N* Immunologic and viral factors in the pathogenesis of systemic lupus erythematosus. Arthritis Rheum *13* 885–894 1970

22 *Talal N & Pillarisetty R* IgM and IgG antibodies to DNA RNA in systemic lupus erythematosus. Clin Immunol Immunopath. *4* 24 1974

23 *Talal N Pillarisetty R. DeHoratius R. & Messner R P* Immunologic regulation of spontaneous antibodies to DNA and RNA. I Significance of IgM and IgG antibodies in SLE patients and asymptomatic relatives. Clin Exp Immunol *25* 377–382 1976

24 *Terasaki P I & McClelland J D* Microdroplet assay of human serum cytotoxins. Nature *204* 998–1000 1964

25 *Terasaki P I., Mottironi V D & Barnett E V* Cytotoxins in disease autocytotoxins in lupus. New Engl. J Med. *283* 724–728, 1970

26 *Wernet P & Kunkel H G* Demonstration of specific T lymphocyte membrane antigens associated with antibodies inhibiting the mixed leukocyte culture in man. Transplant. Proc. *5* 1875–1881 1973

27 *Williams R. C Jr Lies R B. & Messner R. P* Inhibition of mixed leukocyte culture response by serum and γ-globulin fractions from certain patients with connective tissue disorders. Arthritis Rheum. *16* 597–605 1973

28 *Winfield J B Winchester R. J Wernet P Fu S M & Kunkel H G* Nature of cold reactive antibodies to lymphocyte surface determinants in systemic lupus erythematosus. Arthritis Rheum. *18* 1–8 1975

29 *Winfield J B Winchester R. J & Kunkel H G* Association of cold reactive antilymphocyte antibodies with lymphopenia in systemic lupus erythematosus. Arthritis Rheum. *18* 587–594 1975

30 *Ziff M* Viruses and the connective tissue diseases. Ann. Int. Med. *75* 951–958, 1971

cta path microbiol scand. Sect. C, 85 469-472, 1977

PASSIVE TRANSFER OF STREPTOZOTOCIN INDUCED DIABETES MELLITUS WITH SPLEEN CELLS

Studies of Syngeneic and Allogeneic Transfer to Normal and Athymic Nude Mice

KARSTEN BUSCHARD and JORGEN RYGAARD

Pathological-anatomical Institute, Kommunehospitalet,
DK 1399 Copenhagen K, Denmark

Buschard, K. & Rygaard, J. Passive transfer of streptozotocin induced diabetes mellitus with spleen cells. Studies of syngeneic and allogeneic transfer to normal and athymic nude mice. Acta path. microbiol. scand. Sect. C, 85 469-472, 1977

This study shows passive transfer of streptozotocin induced diabetes mellitus in mice. Transplants of spleen cells from BALB/c mice streptozotocin treated for 5 days, induced diabetes in normal BALB/c recipients. Treatment of transplants with anti-theta and complement resulted in significant decrease in the degree of diabetes. Athymic nude mouse recipients of BALB/c background also developed diabetes after transplant of spleen cells from both syngeneic and allogeneic (C57/Bl/6) donors. It is concluded that passive transfer of chemically induced diabetes in mice is practicable, in both syngeneic and allogeneic combinations and that thymus derived lymphocytes are significant in this process.

Key words diabetes mellitus passive transfer T-lymphocytes nude mice streptozotocin.

K. Buschard, Pathological-anatomical Institute, Kommunehospitalet,
DK 1399 Copenhagen K, Denmark

Received 22.viii.77 Accepted 9.x.77

The following describes passive transfer in mice of chemically induced diabetes mellitus with spleen cells. It appears that thymus-derived lymphocytes are significant agents in the process.

Two observations from studies in the athymic nude mouse preceded this passive transfer study (*Buschard et al.* 1976, *Buschard & Rygaard* in press). Firstly diabetogenic virus (encephalomyocarditis virus) did not induce diabetes in the nude, but invariably in normal mice. Secondly streptozotocin regularly induces diabetes in normal mice (*Rerup & Tarding* 1969) but twenty per cent of nude mice remained adiabetic after single dosage of streptozotocin (200 mg/kg BW i.v.) and although all became diabetic after repeated injections (40 mg/kg BW intraperitoneally daily over five days,—*Like & Rossini* 1976) the degree was consistently lower than in normal controls. In consequence, attention was concentrated on the thymus and specifically on thymus-derived lymphocytes.

We used spleen cells, untreated or anti-theta-treated, in our present attempt to create direct evidence (*Milgrom & Witebsky* 1962) for the involvement of the immune response in diabetogenesis.

MATERIALS AND METHODS

Experimental Animals

Donors Thirty three BALB/c/BOM and eleven C57/Bl/6/BOM all normal male 6–8 week old. Nineteen and six respectively were given streptozotocin the others were buffer injected controls.

Recipients Thirty six normal BALB/c and fifty nine nude mice (11th backcross of a gene transfer to BALB/c/BOM) all male 6–8 week old

The distribution in experimental groups and between experimental and control animals can be seen from Table 1 All mice were purchased from Gl. Bomholtgaard Laboratory Animals Breeding and Research Center DK-8680 Ry Denmark. The mice were reared under specified pathogen-free (spf)-conditions. Over the experimental period, animals were kept in pairs in Makrolon® type II cages, at the Pathological-anatomical Institute, Kommunehospitalet. Autoclaved feed pellets (Gl. Bomholtgaard) and sterile drinking water was supplied ad lib

Streptozotocin

Streptozotocin was kindly donated by Upjohn (Kalamazoo Mi., USA) Donors were injected intraperitoneally with streptozotocin, 40 mg/kg BW daily in citrate buffer (pH 4.2) over 5 days, corresponding to a total dose of 200 mg/kg BW (*Like & Rossini 1976*) The occurrence of diabetes in all donors was controlled by a measurement of blood glucose, 248 ± 28 mg/100 ml (± 2 SEM) by day 9 The animals were sacrified on day 12 Control donors were injected with buffer alone

Spleen cells

Spleen cell suspensions were prepared as described elsewhere (*Buschard* in press) A standard transplant of 4×10^7 viable cells in 0.5 ml BSS was injected intraperitoneally into recipients. Control recipients received similar transplants from donors injected with buffer solution

Anti-theta Treatment

Transplants in experimental group 2 and part of 3 were treated with a commercially available anti-theta serum (AKR anti-C3H Searle, High Wycombe, UK) The spleen cells were incubated with anti theta, titer 1 10 at 37 C for 1 hour in the presence of complement (ORAY Bayer) titer 1 40 Following incubation, the cells were resuspended in BBS buffer and washed twice.

Seventeen per cent of the standard preparation of cells (4×10^7) were killed—possible less than the full number of T lymphocytes—and the remaining viable cells were transplanted intraperitoneally into recipients.

Blood Sampling and Testing

Blood for glucose determinations was collected in 25 µl pipettes from the paraorbital venous plexus of non fasting animals on day 0, 3 6 and 11 after transplantation Blood glucose levels were determined spectrophotometrically following incubation with orthotuluidin reagent.

Experimental Design

This study comprises the following experiments

1) *Syngeneic transfer to normal mice* Transfer of untreated spleen cells from normal streptozotocin treated BALB/c donors to normal, untreated BALB/c recipients.
2) *Role of anti-theta sensitive cells* Same design as 1) transplant treated with anti-theta and complement.
3) *Syngeneic transfer to nude mice* Transfer of spleen cells as in 1) and 2) into untreated athymic nude mice of BALB/c background
4) *Allogeneic transfer to nude mice* Transfer of untreated spleen cells from normal, streptozotocin treated C57/Bl/6 donors to untreated nude recipients of BALB/c background.

Statistical Studies

The Mann-Whitney U-test was applied to determine significance.

RESULTS

This study has shown that transplants of spleen cells from mice with streptozotocin-induced diabetes mellitus, induce diabetes in normal and athymic nude recipients, in both syngeneic and allogeneic combinations, and has further demonstrated a decrease in the degree of diabetes after treatment of the transplant with anti theta serum and complement. Detailed results of the 4 experimental groups are given in the table and are briefly described in the following

Syngeneic Transfer to Normal Mice

All experimental group animals (n = 10) became diabetic—blood sugar on day 3 after transplantation 220 ± 24 mg/100 ml. Blood sugar in the control group (n = 8) remained normal 96 ± 18 mg/100 ml The difference is highly significant ($P < 0.01$) Findings were similar on the sixth and eleventh days (see Table 1)

TABLE 1 *Blood Sugar Values in Normal and N de Recipient Mice after Spleen Cell Transf*

Donor	Antitheta complement treated	Recipient	3 days	6 days	11 days
Strep BALB/c	—	(10) BALB/c	(10) 220±24	(10) 268±43	(10) 231±32
Control BALB/c	—	(8) BALB/c	(8) 96±18	(7) 97±13	(7) 93±10
Strep. BALB/c	+	(10) BALB/c	(10) 194±38	(10) 197±21	(10) 206±31
Control BALB/	+	(8) BALB/c	(8) 131±18	(8) 120±12	(7) 107±10
Strep BALB/c	—	(10) BALB/c N	(10) 279±34	(9) 268±51	(9) 270±41
Control BALB/c	—	(8) BALB/ N	(5) 149±21	(5) 136±33	(5) 119±23
Strep BALB/	+	(10) BALB/c Nu	(8) 210±22	(6) 199±25	(6) 223±38
Control BALB/	+	(10) BALB/c Nu	(9) 89±10	(8) 118±24	(7) 121±32
Strep C57	—	(12) BALB/c N	(11) 209±28	(9) 219±31	(9) 229±22
Control C57	—	(9) BALB/c N	(7) 113±22	(5) 112±18	(5) 112±9

A erage blood glucose alues of mice in mg/100 ml + 2 SEM. Number in brackets indicate number of mice in each group Blood sugar alues in recipients for day 0 BALB/c 90±8 mg/100 ml and BALB/c N 82±8 mg/100 ml

Role of Anti-theta Sensitive Cells

The ten recipients of anti-theta and complement treated transplants all developed diabetes, but to consistently lesser degree than those receiving transplants not incubated with anti theta. The difference was statistically significant on the sixth day blood sugar 197 ± 21 *mg/100 ml and* 268 ± 43 *mg/100* ml ($P < 0.01$) The 8 control animals transplanted with normal spleen cells from normal donors, also incubated with anti-theta, showed a slight increase in blood glucose alues, but remained clearly non-diabetic.

Syngeneic Transfer to Nude Mice

All nude mice ($n = 10$) transplanted with spleen cells from (near) syngeneic donors developed diabetes to the same degree as normal recipient BALB/c mice. When the streptozotocin spleen cell transplants were first incubated with anti-theta and complement, the nude mice continued to develop diabetes, but to a lesser degree ($P < 0.02$ on day 3) Nude reaction is thus directly comparable w th that of normal mice in this context. The 5 nude controls showed a slight increase in blood glucose values, but remained clearly non-diabetic

Allogeneic Transfer to Nude Mice

All nude mice of BALB/c background *($n = 12$) transplanted with* streptozotocin spleen cell transplants from C57/Bl/6 donors developed diabetes to the same degree as in the syngeneic study—peak blood sugar 229 ± 22 mg/100 ml by day 11 (compare *with Table 1*)

DISCUSSION

The collected observations of this study establish direct evidence of an immunological component in the process of islet B-cell damage in mice *transplanted* with spleen cells from donors with streptozotocin induced diabetes.

From the findings that anti theta and complement treated spleen cells induce a consistently lesser degree of diabetes we postulate that T-lymphocytes are significant in the diabetes transfer mechanism, and deduce therefrom that the same *T-lymphocytes* are very likely to be involved in the primary pathogenesis.

On the other hand, the fact that the mice in experimental group 2 and part of 3 in spite of the anti-theta treatment, did become diabetic—although to a lesser degree—can be

explained as a dose-response effect. It is known that such anti theta and complement treatment will kill only part of the T-cells. Another possibility is that other elements of the transplant could play a role in the diabetogenesis—independently or in combination with the T lymphocytes. In this context antibodies must be thought of—present in the transplant either in the form of cytophilic antibodies or secreted by transferred antibody producing cells. The possible effect of humoral antibody transfer from diabetic donors is under investigation.

Normal recipients and athymic nude recipients developed diabetes to the same degree This observation in nude mice is of immunopathological interest. In studies of passive cellular transfer of allergic encephalomyelitis and allergic adrenalites in rats by transplants of sensitised lymph node cells, it has been shown that other cells, presumably of recipient origin collaborated in the tissue destruction initiated by the donor lymph node cells (*Werdelin* 1972) Lesions could not be induced if the recipients were first subjected to sub-lethal irradiation probably because a necessary adjunctive recipient cell population was destroyed. Nude mice, in spite of their immunodeficiency disorder are capable of developing passively transferred diabetes when the transplant is spleen cells.

Passive transfer to nude mice is also successful when allogeneic spleen cells are transplanted. With reference to other transplantation studies of e.g normal skin (*Rygaard* 1973) this observations could open the possibility of heterogeneic transfer (including transfer from humans) when the recipients are nude mice This possibility is being investigated.

In a concurrent study one of us (*Buschard* in press) has demonstrated that also virally induced diabetes mellitus can be transferred with spleen cells. These parallel findings directly evidence the involvement of immune phenomena in the pathogenesis of diabetes. They fulfil the fifth of the classical requirements (*Milgrom* & *Witebsky* 1962) of an "auto-immune disorder" the successful transfer of the disease by an antibody containing serum or by immunologically stimulated cells

---

Karsten Buschard is a Research Fellow of the *Michaelsen Foundation Jytte Krammer* and *Inger Bull* gave skillful technical assistance. The study was supported in part by the *Danish Medical Research Council* grant no 512 5732 and 512-6534

REFERENCES

1 *Buschard K* Passive transfer of virus induced diabetes mellitus with spleen cells. Acta path. microbiol. scand. Sect. C in press.

2. *Buschard K* & *Rygaard J* Is the diabetogenic effect of streptozotocin in part thymus-dependent? Acta path. microbiol. scand. Sect. C, in press.

3 *Buschard K Rygaard J* & *Lund E.* The inability of a diabetogenic virus to induce diabetes mellitus in athymic (nude) mice. Acta path. microbiol. scand. Sect. C, *84* 299–303 1976.

4 *Like A A* & *Rossini A A* Streptozotocin induced pancreatic insulitis New model of diabetes mellitus. Science *193* 415–417 1976.

5 *Milgrom F* & *Witebsky E.* Autoantibodies and autoimmune diseases. J.A.M.A. *181* 706–716 1962

6 *Rerup C* & *Tarding, F* Streptozotocin and alloxan diabetes in mice. Eur J Pharmacol. 7 89–96, 1969

7 *Rygaard J* Thymus & self Immunobiology of the mouse mutant nude. F.A.D.L., Copenhagen and J Wiley & Sons, London 1973

8. *Werdelin O* The origin, nature and specificity of mononuclear cells in experimental autoimmune inflammations Thesis. Acta path microbiol. scand. Sect. A, Suppl *232* 1972.

Acta path. microbiol. scand. Sect. C, 85 473–479 1977

EFFECT OF HUMAN LEUCOCYTE MIGRATION INHIBITORY FACTOR (LIF) ON 3, 5'-CYCLIC AMP LEVELS OF PERIPHERAL BLOOD LEUCOCYTES

K. Bendtzen, J. Thode and S. Nistrup Madsen

Laboratory of Clinical Immunology Medical Department TA, Rigshospitalet, University Hospital and Medical Department of Endocrinology F Herlev Hospital, Copenhagen, Denmark

Bendtzen, K. Thode, J and Nistrup Madsen, S Effect of human leucocyte migration inhibitory factor (LIF) on 3' 5-cyclic AMP levels of peripheral blood leucocytes. Acta path. microbiol. scand Sect. C, 85 473–479 1977

The possible involvement of 3' 5-cyclic AMP (cyclic AMP) in the mechanism of action of leucocyte migration inhibitory factor (LIF) was tested. LIF-treated human peripheral blood leucocytes were incubated at 37 C for various times between 0 and 22 h. The concentrations of cyclic AMP in these cultures did not differ from those in controls. Furthermore LIF did not affect the cellular release of cyclic AMP Neither when testing purified neutrophils, which are the prime targets of LIF action, an effect of LIF was found. Cyclic AMP levels decreased with time of incubation, whether testing mononuclear cells (92 per cent lymphocytes) purified neutrophils (98 per cent) or buffy coat cells (72 per cent neutrophils; 22 per cent lymphocytes) However transient and as yet unexplained increase in the cyclic AMP levels of the latter mixed population of cells was manifested within 10 to 30 min of incubation.

Key words. Leucocyte migration inhibitory factor (LIF) leucocyte cyclic AMP neutrophil cyclic AMP lymphocyte cyclic AMP

K. Bendtzen, Laboratory of Clinical Immunology Medical Department TA, Rigshospitalet University Hospital, Tagensvej 18, DK 2200 Copenhagen N Denmark.

Received 22.vi.77 Accepted 10.viii.77

It has been established that 3' 5-cyclic AMP (cyclic AMP) plays a role in lymphocyte activation (18) and lymphokine production (11 12, 14) Evidence has also accumulated that cyclic nucleotides regulate the functions of other important effector cells in the immune response (7) Thus, several neutrophil functions such as phagocytosis, release of lysosomal enzymes, chemotactic responsiveness and spontaneous motility are inhibited by increased levels of cyclic AMP (15 20) and stimulated by increased levels of 3' 5-cyclic GMP (cyclic GMP) (9 20)

The possible involvement of cyclic nucleotides in yet another neutrophil function, the impaired cell migration induced by leucocyte migration inhibitory factor (LIF) has been suggested recently (5 6) The neutrophil response to this lymphokine is significantly depressed by agents which among other effects are capable of increasing intracellular cyclic AMP (6) and cells treated with cyclic AMP and its lipid soluble dibutyryl derivative

escape LIF induced migration inhibition (5) Since the LIF neutralizing effect of cyclic AMP is not merely due to a cyclic AMP induced enhancement of neutrophil migration (5) these findings suggest a role of cyclic AMP in LIF induced migration inhibition

This paper will demonstrate that the involvement of cyclic AMP in the mechanism of LIF action is indirect, since LIF by itself has no significant effect on the cyclic AMP levels of human *neutrophils*

MATERIALS AND METHODS

Production and Assay of Human LIF

The production of LIF by concanavalin A stimulation of peripheral blood lymphocytes and the subsequent Sephadex G-100 column chromatography was carried out as previously described (1) LIF-containing fractions and their control counterparts were pooled lyophilized and stored at — 20 C before use

The LIF activity was determined by the indirect leucocyte migration agarose technique, essentially as detailed previously (4) 22 × 10⁶ peripheral blood buffy coat leucocytes or 15 × 10⁶ peripheral blood neutrophils/90 µl supernatant were tested in 10 µl aliquots for migration under agarose, and the migration index (MI) was determined Mean migration area of cells in LIF rich supernatant/mean migration area of cells in control supernatant. Mean area was calculated by quadruplicate tests.

Preparation of Cells and Supernatants

Buffy coat leucocytes were obtained by dextran sedimentation of heparinized venous blood from healthy adults as described previously (4) The cell population consisted of 72 per cent ± 10 (SD) neutrophils, 22 per cent ± 8 lymphocytes and 6 per cent ± 4 monocytes n = 4 The few erythrocytes contaminating these leucocyte preparations were not removed, since cyclic AMP cannot be formed by non nucleated red blood cells (16)

In some experiments purified neutrophils were prepared as follows

The buffy coat cell layer of dextran sedimented venous blood was diluted twice th Hanks balanced salt solution (HBSS) and layered on an isopaque-ficoll gradient (Lymphoprep ® Nyegaard & Co Oslo Norway) After centrifugation for 40 min at 20 g the cell pellet consisting of leucocytes (98 per cent neutrophils and 2 per cent mononuclear cells n = 3) and approximately an equal number of contaminating erythrocytes wa washed thrice with HBSS before resuspension in LIF rich and control supernatants and subsequent cyclic AMP determinations. The cell layer in the interfase consisting of mainly mononuclear cells (92 per cent lymphocytes, 7 per cent monocytes, 1 per cent neutrophils n = 3) was also isolated, washed three times with HBSS and tested separately see Results.

Lyophilized LIF rich and control supernatants were dissolved in medium TC-199 (Hepes-buffered at pH 7 4 containing antibiotics and 10 per cent heat inactivated horse serum) (TC-199) Two different concentrations were used for each set of experiments (thrice concentrated and unconcentrated, respectively) The presence of LIF activity was assured by testing supernatants on the same leucocyte population as used for cyclic AMP determinations buffy coat cells and neutrophils, respectively

Effect of LIF on Cyclic AMP Levels of Leucocytes

Washed buffy coat leucocytes, neutrophils and lymphocytes were resuspended in 90 µl prewarmed LIF rich and control supernatants and incubated at 37 C in a humidified 5 per cent CO_2 air atmosphere. As a further control, cells were incubated in parallel in 90 µl TC-199 unless stated otherwise. After various times the incubations were terminated by the addition of 100 µl boiling Tris-EDTA buffer (50 mM Tris, 5 mM EDTA ph 7 4) immediately followed by 3 min in a boiling water bath The cyclic AMP-generating and -degrading enzymes were hereby destroyed After centrifugation for 20 min at 400 g supernatants were removed and stored at — 20 C before cyclic AMP assay

This standard procedure was employed for a combined determination of intra and extracellular cyclic AMP In some experiments, however the intracellular and the extracellular cyclic AMP levels were tested separately After incubation at 37 C the cells were separated by centrifugation for 5 min at 220 g, 50 µl supernatant was removed, and 50 µl boiling Tris EDTA buffer was added. The resulting 100 µl solution was then incubated in a boiling water bath for 3 min and tested for cyclic AMP content. The remaining supernatant and cells were added 150 µl boiling Tris-EDTA and processed further as described above

Cyclic AMP Assay

Cyclic AMP was measured with a competitive protein-binding assay as described in detail by *Nutrup Madsen et al.* (13)

RESULTS

Recently LIF has been shown to be an enzyme subjected to irreversible inhibition after challenge with the active site directed

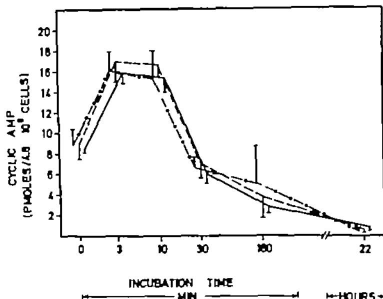


Fig 1 The effects of LIF on the cyclic AMP levels of human peripheral blood buffy coat leucocytes. Results are expressed as means of four to eight different incubations of 4.8×10^6 leucocytes per 90 μl supernatant. Two different concentrations of LIF were used. Vertical bars represent standard deviations. — — • — LIF-rich supernatant — — — Control supernatant. ——— Medium alone.

serine esterase inhibitors di isopropylfluorophosphate and phenylmethylsulfonyl fluoride (2) The LIF molecule was shown to be protected against this inactivation by only two of a wide variety of simple esters, an arginine ester and the phosphodiester bis-p-nitrophenyl phosphate (3) These findings indicate an affinity of the LIF molecule for arginine esters and phosphodiesters Since the mechanism of action of LIF may involve a decrease in neutrophil cyclic AMP concentrations (6) we first tested whether LIF by itself possessed high or low K_m cyclic AMP phosphodiesterase activity LIF rich and control supernatants were incubated at 37 C with 3×10^{-6} M and 3×10 M cyclic AMP After 0 20 40 60, 120 and 180 min of incubation the reactions were stopped by addition of equal volumes of boiling Tris-EDTA buffer and by further boiling for 3 min. These experiments indicated that cyclic AMP phosphodiesterase activity was present neither in LIF rich nor in control supernatants (results not shown)

Experiments were next carried out to determine the effect of LIF on cyclic AMP levels of the rather heterogenous cell population previously used as indicators in the leucocyte migration agarose technique (4) 4.8×10^6 buffy coat leucocytes were incubated for various time periods in 90 μl of either LIF rich supernatant, control supernatant or medium alone (see Fig 1) The presence of LIF activity was confirmed in each experiment by testing the supernatants on the same cell preparation in the agarose assay (MI 0 44 (thrice concentrated) and 0 77 (unconcentrated)) Neither dose of LIF seemed to affect the leucocyte concentration of cyclic AMP as compared to cells maintained in control supernatant or in TC-199 The results, summarized in Fig 1 also revealed a significant but transient increase in cyclic AMP levels followed by a steady decline to almost zero after 22 h of incubation. These alterations were the same whether cells were incubated in LIF-rich control supernatants or medium alone.

The possibility that LIF might act by enhancing the release of cyclic AMP from leucocytes, thus lowering the intracellular

escape LIF induced migration inhibition (5) Since the LIF neutralizing effect of cyclic AMP is not merely due to a cyclic AMP induced enhancement of neutrophil migration (5) these findings suggest a role of cyclic AMP in LIF induced migration inhibition

This paper will demonstrate that the involvement of cyclic AMP in the mechanism of LIF action is indirect since LIF by itself has no significant effect on the cyclic AMP levels of human neutrophils

MATERIALS AND METHODS

Production and Assay of Human LIF

The production of LIF by concanavalin A stimulation of peripheral blood lymphocytes and the subsequent Sephadex G-100 column chromatography was carried out as previously described (1) LIF-containing fractions and their control counter parts were pooled lyophilized and stored at — 20 C before use.

The LIF activity was determined by the indirect leucocyte migration agarose technique, essentially as detailed previously (4) 22×10^6 peripheral blood buffy coat leucocytes or 15×10^6 peripheral blood neutrophils/90 µl supernatant were tested in 10 µl aliquots for migration under agarose and the migration index (MI) was determined Mean migration area of cells in LIF rich supernatant/ mean migration area of cells in control supernatant Mean area was calculated by quadruplicate tests.

Preparation of Cells and Supernatants

Buffy coat leucocytes were obtained by dextran sedimentation of heparinized venous blood from healthy adults as described previously (4) The cell population consisted of 72 per cent ± 10 (SD) neutrophils, 2? per cent ± 8 lymphocytes and 6 per cent ± 4 monocytes n = 4 The few erythrocytes contaminating these leucocyte preparations were not removed since cyclic AMP cannot be formed by non-nucleated red blood cells (16)

In some experiments purified neutrophils were prepared as follows

The buffy coat cell layer of dextran sedimented venous blood was diluted twice with Hanks balanced salt solution (HBSS) and layered on an isopaque-ficoll gradient (Lymphoprep ® Nyegaard & Co., Oslo Norway) After centrifugation for 40 min at 20 g the cell pellet consisting of leucocytes (98 per cent neutrophils and 2 per cent mononuclear cells n = 3) and approximately an equal number of contaminating erythrocytes wa washed thrice with HBSS before resuspension in LIF rich and control supernatants and subsequent cyclic AMP determinations. The cell layer in the interfase consisting of mainly mononuclear cells (92 per cent lymphocytes, 7 per cent monocytes, 1 per cent neutrophils n = 3) was also isolated, washed three times with HBSS and tested separately see Results.

Lyophilized LIF rich and control supernatants were dissolved in medium TC-199 (Hepes-buffered at pH 7 4 containing antibiotics and 10 per cent heat inactivated horse serum) (TC-199) Two different concentrations were used for each set of experiments (thrice concentrated and unconcentrated, respectively) The presence of LIF activity was assured by testing supernatants on the same leucocyte population as used for cyclic AMP determinations buffy coat cells and neutrophils, respectively

Effect of LIF on Cyclic AMP Levels of Leucocytes

Washed buffy coat leucocytes, neutrophils and lymphocytes were resuspended in 90 µl prewarmed LIF rich and control supernatants and incubated at 37 C in a humidified 5 per cent CO_2 air atmosphere As a further control, cells were incubated in parallel in 90 µl TC-199 unless stated otherwise. After various times the incubations were terminated by the addition of 100 µl boiling Tris-EDTA buffer (50 mM Tris, 5 mM EDTA ph 7 4) immediately followed by 3 min in a boiling water bath. The cyclic AMP-generating and -degrading enzymes were hereby destroyed. After centrifugation for 20 min at 400 g supernatants were removed and stored at — 20 C before cyclic AMP assay

This standard procedure was employed for a combined determination of intra and extracellular cyclic AMP In some experiments, however the intracellular and the extracellular cyclic AMP levels were tested separately After incubation at 37 C the cells were separated by centrifugation for 5 min at 220 g, 50 µl supernatant was removed, and 50 µl boiling Tris-EDTA buffer was added. The resulting 100 µl solution was then incubated in a boiling water bath for 3 min and tested for cyclic AMP content. The remaining supernatant and cells were added 150 µl boiling Tris-EDTA and processed further as described above

Cyclic AMP Assay

Cyclic AMP was measured with a competitive protein-binding assay as described in detail by Nist up Madsen et al. (13)

RESULTS

Recently LIF has been shown to be an enzyme subjected to irreversible inhibition after challenge with the active site directed

TABLE 1 *Intra and Extracellular Amounts of Cyclic AMP in LIF-treated Human Peripheral Blood Leucocyte Cultures* [a]

Culture conditions		pmoles cyclic AMP	
Supernatants	Incubation time (min)	Intracellular (in 4.8 × 10^6 cells)	Extracellular (in 90 μl supernatant)
Control	0	10.2 ± 1.5	0
	10	16.0 ± 0.8	1.0 ± 0.2
	30	8.0 ± 0.4	1.0 ± 0.4
	180	6.0 ± 0.8	0.2 ± 0.3
LIF rich	0	9.6 ± 2.0	0
	10	17.0 ± 3.6	1.0 ± 0.4
	30	6.6 ± 1.3	0.7 ± 0.4
	180	8.7 ± 1.3	0.2 ± 0.3

[a] 4.8 × 10^6 buffy coat leucocytes were incubated at 37° C in 90 μl LIF rich or control supernatant. Results are expressed as means of four different incubations ± standard deviation.

TABLE 2 *Amounts of Cyclic AMP in Human Peripheral Blood Mononuclear Cell Cultures* [a]

Culture conditions		pmoles cyclic AMP			
Supernatant	Incubation time (min)	Exp. no. 1	Exp. no. 2	Exp. no. 3	Exp. no. 4
TC-199	0 (Control)	17.0	10.8	24.0	21.9
	10	ND [b]	7.9	11.9	16.9
Per cent deviation from Control			— 27	— 50	— 23

[a] 2.4 × 10^6 mononuclear cells were incubated at 37° C in 90 μl medium TC-199

[b] ND = not determined.

concentration of the nucleotide without affecting the total amount of cyclic AMP in the cell suspension, could not be ruled out by the above experiments. However separate measurements of supernatant cyclic AMP the results of which are shown in Table 1 showed that LIF did not increase the extracellular cyclic AMP levels as compared to controls. A similar decrease in the extracellular nucleotide concentration was observed in both cell suspensions.

The effect of LIF on the level of cyclic AMP in neutrophils, which are the prime targets of LIF action (17) was then tested. Six experiments using three different concentrations of purified neutrophils (4.8 × 10^6 9.6 × 10^6 and 19.2 × 10^6 cells per 90 μl supernatant) and two different concentrations of LIF (MI 0.55 (thrice concentrated) and 0.75 (unconcentrated)) were carried out. Fig 2 shows the pooled data. Again, no significant difference was observed between LIF rich and control supernatants. Furthermore, the pattern of response of the cyclic AMP levels during incubation was the same as that seen with buffy coat cells, except that the early increase in the nucleotide concentration was not found. In these experiments, incubations were not extended beyond 3 hours because only very small amounts of cyclic AMP could be demonstrated in buffy coat cells maintained in culture for longer periods

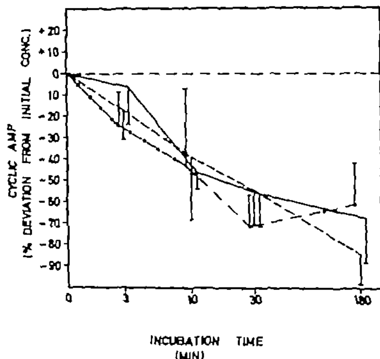


Fig. The effects of LIF on the cyclic AMP levels of human neutrophils. The results of six separate incubations are expressed as the percentage of variation from the neutrophil cyclic AMP concentration found at the beginning of the culture period. The mean initial cell contents of cyclic AMP in experiments using 4.8 × 10⁶, 9.6 × 10⁶ and 19.2 × 10⁶ neutrophils per 90 μl supernatant were 2.0 ± 0.4, 3.3 ± 0.5, and 22.5 ± 2.5 respectively (pmoles ± standard deviation; n = 6). Symbols as in Fig. 1

(Fig. 1) and inhibition of leucocyte migration can regularly be observed as early as 3 hours after incubation in the migration inhibition assay (6).

Finally the possible involvement of lymphocytes, and monocytes, in the observed initial elevation of buffy coat cellular cyclic AMP concentrations was studied. 2.4 × 10⁶ mononuclear cells, 92 per cent of which were lymphocytes, were incubated in 90 μl TC-199. As shown in Table 2, cyclic AMP concentrations repeatedly decreased after 10 min of incubation. Thus, when cultured separately neutrophils and mononuclear cells failed to increase their cyclic AMP levels, whereas mixed cultures of these cell populations constantly increased their cyclic AMP levels after 10 min of incubation under similar conditions (Fig. 1).

DISCUSSION

The experiments presented here were initiated on the basis of two previous findings. First, the synthetic phosphodiester bis-*p*-nitrophenyl phosphate, but not various phosphomonoesters, was capable of retaining LIF activity when LIF rich supernatants were treated with the irreversible inhibitor phenylmethylsulfonyl fluoride (3). The ester not only prevented inhibition of LIF, the protection afforded by the agent was the kind that would be anticipated if the ester and the inhibitor competed for the same site on LIF. This would indicate a possible affinity of the LIF molecule for phosphodiesters such as cyclic AMP, cyclic GMP or both. Second, leucocytes treated with cyclic AMP elevating agents such as isoproterenol, papaverine, dipyridamole and cyclic AMP itself escaped

TABLE 1 *Intra- and Extracellular Amounts of Cyclic AMP in LIF-treated Human Peripheral Blood Leucocyte Cultures*

Culture conditions		pmoles cyclic AMP	
Supernatants	Incubation time (min)	Intracellular (in 4.8 × 10⁶ cells)	Extracellular (in 90 µl supernatant)
Control	0	10 2 ± 1.5	0
	10	16 0 ± 0 8	1 0 ± 0.2
	30	8 0 ± 0 4	1 0 ± 0.4
	180	6.0 ± 0.8	0.2 ± 0.3
LIF rich	0	9 6 ± 2.0	0
	10	17 0 ± 3 6	1 0 ± 0 4
	30	6.6 ± 1.3	0.7 ± 0.4
	180	8.7 ± 1 3	0.2 ± 0.3

4.8 × 10⁶ buffy coat leucocytes were incubated at 37 C in 90 µl LIF rich or control supernatant. Results are expressed as means of four different incubations ± standard deviation.

TABLE 2 *Amounts of Cyclic AMP in Human Peripheral Blood Mononuclear Cell Cultures*

Culture conditions		pmoles cyclic AMP			
Supernatant	Incubation time (min)	Exp. no. 1	Exp. no. 2	Exp. no 3	Exp. no. 4
TC-199	0 (Control)	17.0	10 8	24 0	21.9
	10	ND [b]	7 9	11.9	16.9
Per cent deviation from Control			— 27	— 50	— 23

2 4 × 10⁶ mononuclear cells were incubated at 37 C in 90 µl medium TC-199

[b] ND = not determined.

concentration of the nucleotide without affecting the total amount of cyclic AMP in the cell suspension could not be ruled out by the above experiments. However separate measurements of supernatant cyclic AMP the results of which are shown in Table 1 showed that LIF did not increase the extracellular cyclic AMP levels as compared to controls. A similar decrease in the extracellular nucleotide concentration was observed in both cell suspensions.

The effect of LIF on the level of cyclic AMP in neutrophils, which are the prime targets of LIF action (17) was then tested. Six experiments using three different concentrations of purified neutrophils (4.8 × 10⁶ 9 6 × 10⁶ and 19.2 × 10⁶ cells per 90 µl supernatant) and two different concentrations of LIF (MI 0.55 (thrice concentrated) and 0 75 (unconcentrated)) were carried out. Fig 2 shows the pooled data. Again, no significant difference was observed between LIF rich and control supernatants. Furthermore, the pattern of response of the cyclic AMP levels during incubation was the same as that seen with buffy coat cells, except that the early increase in the nucleotide concentration was not found. In these experiments, incubations were not extended beyond 3 hours because only very small amounts of cyclic AMP could be demonstrated in buffy coat cells maintained in culture for longer periods

3. *Bendtzen K.*, Human leukocyte migration inhibitory factor (LIF) II Partial biochemical characterization of the substrate specificities for the lymphokine. Scand. J Immunol. *6* 133–140, 1977
4. *Bendtzen K., Andersen, V & Bendixen, G.*, An in vitro assay of leukocyte migration inhibitory activity from human lymphocytes stimulated with concanavalin A. Acta Allerg. (Kbh.) *30* 133–149 1975.
5. *Bendtzen, K. & Palit J* *In vitro* modulation of human leucocyte migration and migration inhibitory factor (LIF) activity by cyclic 3 5-AMP and cyclic 3' 5-GMP Acta path. microbiol. scand Sect. C, *85* 78–80, 1977
6. *Bendtzen, K. & Palit J* Modulation of human leucocyte migration inhibitory factor (LIF) by 3' 5-cAMP 3' 5-cGMP and agents known to influence intracellular cyclic nucleotide metabolism. Acta path microbiol. scand. Sect. C, *85* 317–323 1977
7. *Bourne H R., Lichtenstein L. M Melmon K. L., Henney C S Weinstein Y & Shearer G M* Modulation of inflammation and immunity by cyclic AMP Science *184* 19–28, 1974
8. *Boxer L. A Watanabe A. M Rister M., Besch, H R., Allen J & Baehner R. L.* Correction of leukocyte function in Chediak Higashi syndrome by ascorbate N Engl. J Med 295 1041 1045 1976
9. *Estensen R D Hill, H R Quie P G Hogan, N & Goldberg, N D* Cyclic GMP and cell movement N ture 245 458, 1973
10. *George W J Paddock R. J & Ignarro L. J* Cyclic 3 5'-guanosine monophosphate (cGMP) and cyclic 3' 5'-adenosine monophosphate (cAMP) levels in human neutrophils during lysosomal enzyme release Elevation of cGMP by acetylcholine and of cAMP by epinephrine The Pharmacologist *15* 157 1973
11. *Lies, R B & Peter J B* Cyclic AMP inhibition of cytotoxin (Lymphotoxin) elaboration by stimulated lymphocytes. Cell. Immunol. *8* 332 335 1973
12. *Lemaster R., Robson A R. & Koornhof H J* The effects of cyclic AMP on leucocyte inhibitory factor (LIF) production and on the inhibition of leucocyte migration. Clin. exp. Immunol. 24 42–48, 1976.
13. *Vistrup Madsen, S., Badawi I & Skovsted L.* A simple competitive protein-binding assay for adenosine 3' 5-monophosphate in plasma and urine. Acta Endocrinol. (Kbh.) *81* 208–214, 1976.
14. *Pick, E.* Soluble lymphocytic mediators. I Inhibition of macrophage migration inhibitory factor production by drugs. Immunology 26 649–658, 1974
15. *Rivkin I Rosenblatt J & Becker E. L.* The role of cyclic AMP in the chemotactic responsiveness and spontaneous motility of rabbit peritoneal neutrophils. J Immunol. *115* 1126–1134 1975
16. *Robison G. A., Butcher R. W & Sutherland E. W* Cyclic AMP Academic Press, New York and London 1971 p. 29
17. *Rocklin R E.* Products of activated lymphocytes. Leukocyte inhibitory factor (LIF) distinct from migration inhibitory factor (MIF) J Immunol. *112* *1461–1466, 1974*
18. *Watson, J.*. Cyclic nucleotides as intracellular mediators of B cell activation. Transplant. Rev 23 225–249, 1975.
19. *Woo Y T & Manery J F.* Cyclic AMP phosphodiesterase activity at the external surface of intact skeletal muscle and *stimulation* of the enzyme by insulin. Arch. Biochem. Biophys. *154* 510–519 1973
20. *Zurier R. B., Weissmann G., Hoffstein S Kammerman, S & Tai, H H* Mechanisms of lysosomal enzyme release from human leucocytes. II. Effects of cAMP and cGMP autonomic agonists, and agents which affect microtubule function. J Clin. Invest. *53* 297–309 1974

LIF induced migration inhibition (5 6) This suggests that a decrease in leucocyte cyclic AMP concentrations or an increase in cellular levels of cyclic GMP may be involved in the mechanism of LIF action The experiments reported herein investigate the former possibility

A direct esterolytic effect of LIF on cyclic AMP can be ruled out since experiments designed to test both high and low K_m cyclic AMP phosphodiesterase activity of LIF were convincingly negative. Moreover competition experiments indicate that only bis-p-nitrophenyl phosphate and not cyclic AMP protect LIF against the enzyme inhibitor phenylmethylsulfonyl fluoride (*Bendtzen* unpublished observations) Although these experiments both performed in a cell free system, provide strong evidence against a possible LIF affinity for cyclic AMP they do not rule out an effect of LIF on the cellular cyclic AMP system.

As shown in this paper cyclic AMP levels in LIF treated leucocytes did fall during incubation. However no differences were observed between LIF treated and control cells, indicating that LIF by itself did not affect the leucocyte cyclic AMP system, and that the fall in cyclic AMP during culture might be due to lack of oxygen or hormonal stimulators in the medium. From a practical point of view the marked decrease in leucocyte and neutrophil cyclic AMP during incubation at 37 C deserves attention. Thus, when testing basal levels of cyclic AMP in leucocytes it seems extremely important to standardize the time at which measurements are carried out, and it is quite possible that the reported wide variations in neutrophil cyclic AMP levels are due to differences in time elapsed between isolation of the cells and actual measurements of the nucleotide (8 10 20) These considerations are further emphasized by the marked initial increases in cyclic AMP concentrations observed when testing buffy coat leucocytes. Interestingly this transient doubling in cyclic nucleotide levels could not be reproduced in cultures of either purified neutrophils or mononuclear cells. It was apparently not density dependent, since neither mononuclear cells (2.4×10^6 cells/ml) nor neutrophils (4.8 to 19.2×10^6 cells/ml) showed this phenomenon. At present we can offer no reasonable explanation for these unexpected findings.

A possible influence of LIF on the release of cyclic AMP from the leucocytes without affecting their capacity to generate cyclic AMP could be ruled out. In both experimental and control cell systems the extracellular amounts of cyclic AMP were always very small. Furthermore, a steady decline in extracellular cyclic AMP levels during incubation was observed, supporting the findings of *Woo & Manery* (19) that cyclic AMP degrading phosphodiesterase is present on cell surfaces. This enzyme activity however could not be due to LIF since no difference between LIF treated and control cells was seen. This also confirmed our preliminary findings that LIF itself was not capable of degrading cyclic AMP

In summary the LIF induced inhibition of leucocyte migration is probably not due to a direct effect of LIF on cyclic AMP concentrations of neutrophils, although the possibility that LIF may induce changes in cyclic AMP levels in certain subcellular sites of the neutrophils which may be of particular importance for cell motility cannot be entirely ruled out. This conclusion, and those of previous reports, however still leaves open a role of cyclic GMP as a putative 'second messenger' of LIF action. This possibility is presently being investigated.

This work was supported by the *Danish Medical Research Council.*

REFERENCES

1 *Bendtzen K* Inhibition of human leukocyte migration inhibitory factor (LIF) by α-L-fucose. Acta Allerg. (Kbh.) *30* 327–338, *1975*
2. *Bendtzen K* Human leukocyte migration inhibitory factor (LIF) I Effect of synthetic and naturally occurring protease inhibitors. Scand J Immunol *6* 125–131 1977

TABLE 1. *Proportions of Various Human Sera Precipitating Human Aggregated IgG*

	No. of sera tested	No. of sera precipitating aggregated IgG
Sera from patients with rheumatoid arthritis		
Waaler Rose test titre $\geq$ 1:64	50	48 (96)
Waaler Rose test titre 1:16–1:32	6	6 (100)
Waaler Rose test titre $<$ 1:16	8	4 (50)
Sera from patients with juvenile rheumatoid arthritis	14	3 (21)
Sera from blood donors	52	2 (14)

Values in *parentheses denote the percentages of sera precipitating.*

wells at a distance of 4–6 mm between the edges were cut. 15–25 µl of undiluted serum and 5–7 µl of aggregated IgG were used. Most precipitation lines became visible after 1–2 days at 4° C or 22° C but the plates were also inspected after 6–8 days.

Human sera. Sera from 130 human subjects were selected among specimens sent to our laboratory: sera from 50 patients with rheumatoid arthritis and a Waaler-Rose test titre of $\geq$ 1:64 (11), sera from patients with a clinical diagnosis of rheumatoid arthritis and a Waaler-Rose test titre of 1:16–1:32 *(6 sera)* or $<$ *1:16 (8 sera)*, sera from 14 patients with juvenile rheumatoid arthritis and sera from 52 blood donors. All sera were heated at 56° C for 30 min before testing.

2-mercaptoethanol (2-ME) treatment. 500 µl serum was incubated with 50 µl of 2-ME (KEBO AB, Stockholm) 1.0 M for 2 h at 22° C and subsequently dialysed over-night at 22° C against PBS. As described by *Reimink et al.* (7) dialysis could be omitted: precipitability of aggregated IgG was not affected by the presence of 2-ME.

Results

The capacity of human sera to precipitate aggregated IgG was correlated to a positive Waaler-Rose test, though not to the degree of titre elevation (Table 1). Fifty-four of 56 sera with a titre of $\geq$ 1:16 gave rise to precipitation lines, whereas only 4 of 8 rheumatoid arthritis sera, with a titre of $<$ *1:16*, did so. *Precipitation* lines were obtained with 3 of 14 sera from juvenile rheumatoid arthritis patients. Only 2 of 52 blood donors' sera gave rise to precipitation. The sera from patients with rheumatoid arthritis and a Waaler-Rose test titre of $\geq$ 1:16 thus precipitated aggregated IgG significantly more often than did blood donor sera ($p < 0.01$). Furthermore, significantly more rheumatoid arthritis sera *having a titre of* $<$ *1:16* precipitated than did blood donor sera ($p < 0.01$; Chi-square test, exact method). No such difference was found between the juvenile rheumatoid arthritis sera and the blood donor sera (p = 0.054). All sera that gave rise to precipitation lines when tested with the aggregates obtained by Sephadex-filtration did also give lines with the aggregated IgG obtained by sonication; however three *sera reacted only with the latter.*

The capacity to agglutinate sensitized sheep red cells was abolished by 2-ME treatment of 16 investigated sera with a Waaler-Rose test titre of $\geq$ *1:64.* 5 of the sera still precipitated aggregated IgG; 3 of these initially exhibited two precipitation lines one of which persisted after 2-ME treatment (Table 2).

TABLE 2. *16 Rheumatoid Arthritis Sera (Waaler-Rose Titre* $\geq$ *1:64). Influence of 2-ME Treatment on Precipitability by Aggregated IgG*

Precipitation abolished	11 (69 %)
Precipitation not affected	2 (13 %)
One *of two original precipitation* lines abolished	3 (19 %)

Discussion

Attempts have been made earlier to demonstrate precipitating anti-immunoglobulin antibodies in the sera of patients with rheumatoid arthritis. The Cohn fraction II used in the FII tube precipitation test, described in *1956* (2) was later shown to consist of aggregates of gammaglobulin with S_{20} constant as high as 40 (1). *Winchester* (13) and *Theofilopoulos et al.* (9) determined IgG "rheumatoid factor" in pepsin-digested sera by single radial immunoprecipitation in gel, containing aggregated IgG; only *few of the sera* precipitated after digestion.

BRIEF REPORTS

A SIMPLE METHOD FOR DEMONSTRATING GEL-PRECIPITATING HUMAN ANTI IMMUNOGLOBULIN ANTIBODIES

Claes Schalén and Poul Christensen

The Institute of Medical Microbiology
University of Lund Sweden

Schalén, C. & Christensen, P A simple method for demonstrating gel-precipitating human anti-IgG antibodies. Acta path. microbiol. scand. Sect. C, 85 480–482 1977

The use of aggregated human IgG for the detection of anti-immunoglobulin antibodies by double immunodiffusion in gel is described. Of rheumatoid arthritis sera, 54 of 56 sera having a Waaler Rose test titre of ≧ 1 16 contained antibodies to aggregated IgG in contrast to only 2 of 52 sera from blood donors. Furthermore, after 2-mercapto-ethanol treatment anti IgG was still demonstrated in 5 of 16 sera.

Key word Anti immunoglobulins.

C. Schalén, Institute of Medical Microbiology University of Lund,
Sölvegatan 23 S-223 62 Lund Sweden.

Received 15.x.77 Accepted 15.x.77

The Waaler Rose agglutination test (11) demonstrates anti immunoglobulins of the IgM class in sera from patients with rheumatoid arthritis. Various techniques have been employed for the demonstration of anti Ig of the IgG class, including tube precipitation of aggregated human IgG (4 8) and the use of normal rabbit IgG as an immunosorbent (3 10) Furthermore, anti Ig of IgG class were shown in pepsin-digested sera by radial immunodiffusion in gel containing aggregated IgG (9 13)

Sera containing rheumatoid factors have been used to demonstrate aggregated IgG and immune complexes by double diffusion-in-gel techniques (6 12) The present paper describes a simple procedure for demonstration of anti-immunoglobulins of IgM as well as of IgG class utilizing precipitation in gel of aggregated IgG

Materials and Methods

Heat-aggregated human IgG Three ml of 30 mg/ml concentrated commercial human IgG (AB Kabi, batch No. 44791) in phosphate-buffered saline (PBS 0 03 M phosphate, 0 12 M NaCl, pH 7 2) was incubated at 63 C for 12 min. The sample was then centrifuged at 3000 *g* for 20 min. The sediment was discarded and the supernatant filtered through a Sephadex G 200 column (2.5 × 90 cm filtration rate 12 ml/h fractions 3.5 ml) (Pharmacia Fine Chemicals, Sweden) Two major peaks were obtained The first, occurring in the void volume was concentrated to 5 1–6.3 mg/ml on an ultrafiltration cell (Diaflo XM 50) and stored at −80 C until used in gel diffusion experiments. The second peak corresponding to non-aggregated IgG was discarded.

For some experiments, the unwashed sediment obtained on centrifugation of heated IgG was suspended in PBS and sonicated (Ultrasonic power unit type 7685/2 MSE, London) for 30 min during cooling in an ice bath. The supernatant resulting from centrifugation at 3000 *g* for 30 min, was used in the gel-diffusion experiments at a concentration of 10–12 mg/ml.

Protein concentrations were determined by a modification of Folin s method (5)

Gel precipitation tests. Analysis by the double diffusion technique was performed in 0 6 per cent agarose (Miles Ltd., England) in PBS. Five ml gel was poured over an 8 × 8 cm glass plate and

Acta path. microbiol. scand. Sect. C, 85 483–486, 1977

CONCANAVALIN A INDUCED ACTIVATION OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS MEMORY LYMPHOCYTES INTO SPECIFICALLY CYTOTOXIC T CELLS

Ole Marker Allan Randrup Thomsen and Grethe Thérese Andersen

Institute of Medical Microbiology University of Copenhagen,
Juliane Maries Vej 22, DK-2100 Copenhagen, Denmark

Marker O Thomsen, A. R. & Andersen, G. T Concanavalin A-induced activation of lymphocytic choriomeningitis virus memory lymphocytes into specifically cytotoxic T cells. Acta path. microbiol. scand. Sect. C, 85 483–486, 1977

When spleen cells, which have been primed to Lymphocytic Choriomeningitis (LCM) virus during a primary infection several months previously are stimulated in *vitro* with Con A, highly specific secondary cytotoxic effector cells are generated. The degree of cytotoxicity revealed by such Con A-stimulated cells is higher than that of non-incubated spleen cells harvested nine days following the primary infection, and the effect is totally inhibited by anti-theta serum plus complement treatment of the effector cells immediately before the cytotoxic test

Key words Concanavalin A lymphocytic choriomeningitis virus memory cells T lymphocyte-mediated cytotoxicity

O Marker Institute of Medical Microbiology University of Copenhagen,
Juliane Mariesvej 22, DK 2100 Copenhagen, Denmark.

Received 26.ix.77 Accepted 26.ix.77

It is well established that stimulation with both allografts and viral antigens leads to cell-mediated immune responses involving the appearance of specific cytotoxic T lymphocytes In the majority of systems investigated, maximal cytotoxicity is found six to ten days following antigen stimulation, and after a gradual decline it falls off to very low levels (2, 3, 4 5 7 8, 14 15) Generally responses of lymphocytes from such primed animals to the homologous antigen leads *to* generation of specific effector cells demonstrating immunological memory T cells (5 9, 10) Results obtained in an allogeneic system show that irrespective of whether priming or restimulation takes place in vivo or in vitro such T cell-mediated secondary responses are higher than those seen during the primary stimulation (2, 6, 11) It has been shown recently that splenocytes primed in vivo to an allograft generate cytotoxic T cells when exposed to Concanavalin A (Con A) in culture but without the presence of the homologous antigen. The specificity of the cytotoxic effect shown by these cells and the degree of their efficiency corresponds to the results obtained by stimulation of the primed lymphocytes with the homologous antigen (1) In the light of these results, the present work was undertaken to test whether memory cells primed to LCM virus could be activated in vitro by Con A into secondary cytotoxic T lymphocytes. This might possibly reveal the cytotoxic potential of LCM virus-primed memory cells.

Materials and Methods

Virus. LCM virus of the Traub strain—produced, employed and stored as described previously (13)—was used throughout the study Virus titrations were carried out by intracerebral (i.c.) inoculations into ordinary 12 14 g white Swiss mice Administered by this route, LCM virus pro-

In the present investigation, 54 out of 56 sera from patients with rheumatoid arthritis and a Waaler Rose test titre ≧ 1 16 precipitated aggregated IgG in the double immunodiffusion procedure. Judging from the results of 2 ME treatment the precipitating antibodies were of the IgM class in most of the sera and of the IgG class in a lesser proportion. Occasionally antibodies of both these Ig-classes were present. This is in accordance with other reports (9 12)

The applicability of the method described in this paper for the demonstration of anti Ig was dependent on the use of aggregated IgG in a concentration of 3–12 mg/ml and 25–30 µl of rheumatoid arthritis sera. For optimal test conditions it is furthermore important to remove native IgG from the preparation of aggregated IgG Non-aggregated IgG interferes with the precipitation of aggregated IgG by rheumatoid factors as demonstrated by *Christian* (1) in tube experiments.

The method described is not as sensitive as the method utilizing radiolabelled aggregated IgG (4) for the demonstration of anti immunoglobulins in tube precipitation technique. However by the demonstration of anti IgG by the well known immunodiffusion technique the method will make it possible to reveal the participation of anti-IgG in other antigen antibody systems in for instance rheumatoid arthritis sera. By the method described it is possible to demonstrate anti-Ig of the IgG class which is of clinical importance as the presence of such antibodies in rheumatoid arthritis seems to be correlated to signs of vasculitis (9)

References 1 *Christian C L.* J exp. Med. *108* 139–157 1958 —2. *Epstein W Johnson A & Ragan C.*. Proc Soc. Exper Biol. & Med. *91* 235–237 1956 —3 *Hay F C., Nineham L J & Roitt I M* Br Med. J *3* 203–204 1975.—4 *Hällgren R & Wide L.* Ann. Rheum. Dis. *36* 21–29 1977 —5 *Lowry O H Rosebrough N J Farr L. A & Randall R J* J Biol. Chem. *193* 265–275 1951 —6. *Munthe E. & Natvig, J B* Clin. Exp. Immunol. *8* 249–262, 1971 —7 *Reesink H W van der Hart M & van Loghem J J* Vox. Sang. *22* 397–407 1972.—8. *Schrohenloher R* J Clin. Invest. *45* 501–512, 1966.—9 *Theofilopoulos A N Burtonboy G LoSpalluto J J & Ziff M* Arthr Rheum. *17* 272 284 1974 —10. *Torrigiani G & Roitt I M* Ann. Rheum. Dis. *26* 334–340 1967 —11 *Waaler E.* Acta path. microbiol. scand. *17* 172–188, 1940.—12 *Winchester R J., Kunkel H G & Agnello V* J exp Med *134* 286–295 1971 —13 *Winchester R J* Ann N Y Acad Sci *256* 73–81 1975

TABLE 1 *Cytotoxic Effect of Con A-stimulated and Non-stimulated LCM Virus Primed Splenocytes*

Effector splenocytes	Con A stimulation	*In vitro* incubation	Target cells	Ratio effector/targets	Cytotoxic index ± SD	LU[illegible]/10^6 cells
9 days	—	No	Infected	25	100	
9 days	—	No	Infected	12.5	82 ± 1.7	3.94
9 days	—	No	Infected	6.25	41 ± 1.8	
9 days	—	N	Uninfected	25	3.2 ± 0.6	
60–90 days	+	Yes	Infected	25	100	
60–90 days	+	Yes	Infected	12.5	100	8.63
60–90 days	+	Yes	Infected	6.25	87 ± 1.8	
60–90 days	+	Yes	Uninfected	25	14 ± 1.4	≦ 0.61
60–90 days		Yes	Infected	25	6.7 ± 1.5	≦ 0.54
60–90 days	—	Yes	Uninfected	25	0	
Normal spleen cells	+	Yes	Infected	25	0	
Normal spleen cells	—	Yes	Infected	25	0	
Normal spleen cells	+	Yes	Uninfected	25	0	
Normal spleen cells	—	No	Infected	25	0	

primed to both LCM virus and alloantigens during Con A stimulation develop such cells indicates similarities between the mechanisms of Con A stimulation on the one hand and restimulation with the homologous antigen on the other. Moreover it is claimed by *Bonavida* (1) that, as regards both the kinetics and the magnitude of the cytotoxicity obtained, the two ways of developing secondary cytotoxic cells lead to the same result. However, there is also experimental evidence which indicates differences. Thus it has been demonstrated by *Tartof & Fitch* (16) in an allogeneic system that, even though the cytotoxic efficiency after Con A stimulation was similar to that resulting from alloantigen restimulation, the kinetics were consistently found to be different. *Furthermore, that author found that cytotoxic cells* develop independently of DNA synthesis during alloantigen restimulation, whereas the generation of secondary cytotoxic cells induced by Con A does not take place in the presence of the DNA synthesis inhibitor Cytosine Arabinoside.

In the LCM virus system, *in vitro* stimulation of primary cytotoxic cells has not been achieved as yet. However *in vitro* antigen-induced generation of secondary cytotoxic cells has been studied by *Dunlop & Blanden* (9) and *Dunlop et al.* (10). They demonstrated that the cytotoxicity is weak after two days of antigen restimulation, but climbs to a maximum at day five, at which time it is substantially stronger than that obtained with early immune cells (9). Since the results of the present paper show that memory cells stimulated for 48 hours with Con A reveal very high levels of cytotoxicity this may also indicate differences between the two stimulatory mechanisms, although the resulting cells in both cases prove to be T cells (10). These discrepancies need more clarification, and for this purpose investigations are at present being carried out in our laboratory.

The low degrees of cytotoxicity revealed by non-stimulated late immune cells was observed in all experiments in this series. This observation was expected, since previous results showed an analogous effect of spleen cells harvested about 7–8 weeks after primary LCM virus infection of the host animal (14, 15).

The weak cytotoxic effect revealed by Con A-stimulated late immune cells against non-infected targets in one experiment, and by normal Con A stimulated spleen cells against both infected and non-infected target cells in another seems difficult to explain. It cannot be excluded, however, that *minimal quantities of Con A* still adhering to the effector cells may account for these findings. Only experiments which include specific measures to avoid the presence of Con A in the cytotoxic assay may clarify this. In this connection, it can be mentioned that a similar autocytotoxic effect in an allogeneic system has been described recently (1). *The in vivo effects* of both early immune and late immune cells primed to LCM virus have been described in previous publications (17, 18). An investigation into the *in vivo* effects of Con A-stimulated late immune cells might prove to be of interest, and experiments are at present in progress in our laboratory.

References: 1 *Bonavida, B.*, J. exp. Med. *145* 293–301 1977.—2. *Brunner K. T. & Cerottini, J.-C.*, In *Amos B* (Ed.) Progress in Immunology. First International Congress of Immunology. Academic Press, New York & London, 1971 p. 385–398.—3. *Brunner K. T., Mauel, J., Cerottini*

duces a fatal disease. Virus titres were expressed as LD_{50}/0 03 ml If given intraperitoneally the virus causes no disease but renders the animals immune.

Mice Highly inbred C3H mice were used in all experiments.

Early immune cells were spleen cells harvested nine days after intraperitoneal (i.p) inoculation of 10^3 LD_{50}s of LCM virus.

Late immune cells were spleen cells harvested 60–90 days after i.p inoculation of 10^3 LD_{50}s of LCM virus.

Con A stimulation. Single cell suspensions of spleen cells were washed and resuspended in RPMI 1640 medium with 10 per cent Foetal Bovine Serum (FBS) and 5×10^{-5} M 2-mercaptoethanol, and placed in 50 ml Falcon tissue culture flasks. Each flask contained 10 ml cell suspension with a concentration of 3×10^6 cells/ml Con A (Concanavalin A Pharmacia Fine Chemicals AB, Uppsala, Sweden) was added to each flask to a final concentration of 1 µg/ml and the flasks were then incubated for 48 hours in a humid atmosphere containing 3.5 per cent CO_2. Cells to be incubated but not Con A stimulated were treated in exactly the same way except that no Con A was added After incubation, the cells were pelleted and resuspended in fresh Minimum Essential Medium Eagle (MEM) with 10 per cent FBS and counted for viability The number of viable cells harvested was 50–70 per cent of the number originally added to the flasks.

The cytotoxic assay was a slight modification of the ^{51}Cr release assay already described (14) Target cells were always L cells infected with LCM virus as described previously (14) or non-infected for controls. Effector cells were splenocytes from normal mice or from mice infected with LCM virus at various times before the spleen was harvested Post infection L cell suspensions were labelled with 30 µCi $^{51}Cr/10^6$ cells for 30 minutes at 37 C washed five times and resuspended at a concentration of 10^5 cells/ml all in MEM plus 10 per cent FBS Suspensions of spleen cells were prepared in MEM plus 10 per cent FBS as described earlier (14) and adjusted to a concentration of 2.5×10^6 viable cells/ml 2 ml of target suspension was mixed with 2 ml of effector cell suspension for each of three effector/target ratios of 25 12.5 and 6 25 0 7 ml of the mixture was added to five plastic tubes for each ratio The tubes were incubated for 18 hours at 37 C in a 3.5 per cent CO_2-containing atmosphere Following incubation, the tubes were centrifuged at 400 G The supernatant was transferred to counting vials with 0.500 ml per vial, and counting was made in a gamma counter The results were expressed as cytotoxic indices (CI) and the number of lymphoid cells corresponding to 50 per cent lysis was defined as one lytic unit (LU_{50}) The CI and the number of $LU_{50}/10^6$ cells were calculated as described previously (12)

Anti-theta serum was C3H anti AKR thymocyte serum. It was produced, tested and employed as described earlier (17 18)

Results

Spleen cells from LCM virus-primed C3H mice 60–90 days following i.p. inoculation of virus (late immune cells) and spleen cells from normal mice of the same strain, were incubated for 48 hours with or without Con A and tested for cytotoxicity in the ^{51}Cr release assay The targets were L cells either non-infected or infected with LCM virus. In the same assay were also included spleen cells from mice infected with $10^3 LD_{50}$ of LCM virus nine days prior to the experiment (early immune cells) and normal spleen cells, neither of which had been incubated *in vitro* before the test. The results of one of the experiments in this series are given in Table 1 As was expected, early immune cells showed high degrees of cytotoxicity against infected cells. Late immune cells stimulated with Con A also proved to be strongly cytotoxic to LCM virus-infected target cells, even more so than the cells from the acutely infected animals. When tested against non infected L cells, early immune cells showed practically no cytotoxicity However immune cells stimulated with Con A caused a ^{51}Cr release greater than that seen with normal spleen cells. The specific cytotoxic effect of Con A-stimulated late immune cells and early immune cells was completely abrogated when the effector cells were treated with anti-theta serum and complement prior to the ^{51}Cr release assay

Late immune cells incubated for 48 hours without Con A revealed modest but definite cytotoxicity when tested against *infected targets,* whereas this effect was not seen when the targets were non-infected L cells.

In contrast to the findings with late immune cells, no cytotoxicity was observed when normal spleen cells stimulated with Con A were tested against syngeneic LCM virus-infected or non-infected L cells In one experiment from this series, however Con A-stimulated normal spleen cells proved to be slightly cytotoxic against both infected and non-infected targets. Normal splenocytes incubated for 48 hours without Con A did not release more ^{51}Cr from infected target cells than normal non-incubated spleen cells.

Discussion

The results in this paper show that memory spleen cells primed to LCM virus generate specific secondary cytotoxic T cells when stimulated *in vitro* with Con A. The fact that lymphocytes

J-C & *Chapuis B.*, Immunology *14* 181–196 1968.—4 *Brunner K. T.*, *Manel J Rudolf H* & *Chapuis B.* Immunology *18* 501–515 1970 —5 *Canty G T Wunderlich J R* & *Fletcher F* J Immunol. *106* 200–208 1971 —6 *Cerottini J C Engers H D MacDonald H R* & *Brunner K T* J exp Med. *140* 703–717 1974 —7 *Cole G A Prendergast R.A* & *Henney C S* In *Lehmann-Grube F* (Ed.) Lymphocytic Choriomeningitis Virus and other Arenaviruses, Springer Verlag Berlin, Heidelberg New York, 1973 p 61–71 —8 *Doherty P C Blanden R V* & *Zinkernagel R M* Transplant. Rev 29 89–124 1976 —9 *Dunlop M B C* & *Blanden R. V* Immunology *31* 171–180 1976 —10 *Dunlop M B C Doherty P C Zinkernagel R M* & *Blanden R V* Immunology *31* 181–186 1976 —11 *MacDonald H R. Engers H D Cerottini, J-C.* & *Brunner K T* J exp Med. *140* 718–730, 1974 —12 *Marker O* & *Andersen G T* Acta path. microbiol. scand Sect. C, *84* 447–454, 1976 —13 *Marker O Andersen G T* & *Volkert M* Acta path. microbiol. scand. Sect. C, *84* 23–30 1976 —14 *Marker O* & *Volkert M.*, J exp Med. *137* 1511–1525 1973 —15 *Marker O* & *Volkert M* In *Lehmann-Grube F* (Ed.) Lymphocytic Choriomeningitis Virus and other Arenaviruses, Springer Verlag Berlin, Heidelberg, New York, 1973 p 207–216.—16. *Tartof D* & *Fitch F W* J Immunol. *118* 35–42, 1977 —17 *Volkert M Bro-Jorgensen K. Marker O Rubin B.* & *Trier L.* Immunology *29* 455–464 1975 —18 *Volkert M., Marker O* & *Bro-Jorgensen K* J exp Med. *139* 1329–1343 1974